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FURTHER STUDIES ON THE EFFECT OF DESOXYCORTICOSTERONE ACETATE ON EARLY PREGNANCY[1,2]

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IN A previous report, Burdick and Konanz (1941) showed that daily injections of 2 mg. of desoxycorticosterone acetate (DCA), when begun the day of mating, prevented the implantation of mouse embryos. Implantation took place, however, if the injections were delayed until the second day after mating. There was some evidence that after the injections had been started, pregnancy progressed normally and was maintained, apparently, only as long as the females received the exogeneous supply of desoxycorticosterone acetate.

This present investigation was undertaken to determine the accuracy of the latter observation and to gather more detailed information on the following points. 1) The effect of DCA on the progress of early pregnancy. 2) The effects of withholding DCA after a series of daily injections: a) On the uterus and vagina; b) On the embryonic tissues; c) On the corpora lutea of pregnancy; d) On subsequent ovulation. 3) The effect of DCA on non-pregnant metestrous mice. 4) The effect of sesame oil on early pregnancy.

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[2] Supplies of desoxycorticosterone acetate were generously provided by Schering Corporation (Cortate) and Ciba Pharmaceutical Products, Inc. (Percorten).

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[4] Now Ruth T. Rogers, M.D., Daytona Beach, Fla.

PROCEDURE

Albino mice of the Rockland strain were used in these experiments. Mating was determined by the presence of the vaginal plug, and the day of the plug was counted the first day of pregnancy. The animals received subcutaneous injections for a period of 4 to 6 days, and in all cases treatment was begun later than the first day of pregnancy so as not to interfere with the implantation of embryos. Approximately 180 mice were used in this study, but only a few animals, together with certain experimental data and results typical of each series, are shown in the accompanying table. The reproductive tracts of one series of animals (Table 1, Part A) were examined within 24 hours after the last injection, while other animals (Table 1, Part B) were not examined until an interval of one or more days had elapsed after the last injection. Experience had proven that the presence of blood in the vagina, 42 to 60 hours after the last injection, was an indication that the embryos were dead. This show of blood, whether scanty or copious, is called the "abortion sign," and should not be confused with that of the "placental sign" which usually appears on the twelfth day of normal pregnancy in this strain of mice.

A control series was used to determine whether blood would appear in the vagina of non-pregnant animals after the daily injections had been stopped. Metestrous mice received several daily injections of DCA, after which they were examined every two hours for vaginal blood. These observations began 30 hours after the last injection and continued at two-hour intervals until at least 72 hours had elapsed.

Another control series of pregnant mice was injected with sesame oil, which was the solvent for the DCA, to find out if this substance had any deleterious effect on pregnancy.

Before summarizing the results and observations gathered from this investigation, it will be necessary, for the sake of further comparisons, to describe briefly the uteri and embryos of 7- to 12-day old normal pregnant mice. More complete descriptions of early embryology have been given by Snell (1941) and Venable (1939) for the mouse, and by Stafford (1939) for the rat.

It will be recalled that the normal mouse blastocyst becomes attached to the antimesometrial wall of the uterus $4\frac{1}{2}$ to 5 days after fertilization. Soon the uterus develops a decidual swelling which eventually surrounds the embryo. As the decidua increases in diameter, it touches and grows fast to the opposite mesometrial wall where first the decidua basalis and then the placenta forms. The uterine lumen is thus horizontally partitioned by the deciduae into a series of compartments. Free blood from the sinuses around the deciduae accumulates in the lumen between the deciduae, but this cannot escape into the vagina except for a trace from the most posterior decidua in each horn. On approximately the eleventh day of pregnancy, each decidua becomes separated from the antimesometrial wall to which it was first attached. This separation reopens the uterine lumen from end to end, thereby allowing some of the accumulated free blood between the deciduae to escape into the vagina, where it is known as the "placental sign." As the deciduae develop, the intact uterus of the normal pregnant mouse exhibits a beaded appearance with distinct blood vessels filled with bright cherry-red blood between the "beads."

On the ninth or tenth day, wide streaks or bands are visible through the uterine wall. Each bead typically shows a transverse central light band bordered on each side by a pink stripe. Often an area of free blood can be seen in the lumen between the implants. Upon dissection, the light band was found to be the line of endometrial attachment of the implant, and it consisted of the decidua capsularis and an area of smooth,

TABLE 1. EFFECTS OF DCA ON EARLY PREGNANCY

Animal number	Treatment, 1 mg. or 2 mg. DCA daily	Days to autopsy A.V.P.[1]	Comments
	Part A. Animals Sacrificed While Receiving DCA		
44	P[2]/—/2/2/2/2/2/2/A[3]	9	Corpora lutea deep pink. Tracts vascular with bright red blood in wide uterine vessels. Implants apparently normal.
138	P/—/2/2/2/2/2/2/2A	9	Corpora lutea normal. Tracts vascular. Embryos apparently normal.
42	P/2/2/2/2/2/2/2/2/A	10	Corpora lutea vascular but not deep red. Tracts vascular and normal. Ten normal embryos with hearts beating.
43	Same as No. 42	10	Corpora lutea pink, not deep red. Some blood in lumen which is normal for this period. Normal embryos with beating hearts.
39	P/—/—/—/2/2/2/2/2/2/A	11	Very small corpora albicantia. Six implants with bright cherry-red blood. Normal except that embryos have reached only 7-day old stage. Hearts not yet developed. Had DCA kept pregnancy from terminating?
	Part B. Autopsies Delayed		
19	P/—/1/1/1/1/1/—/—/A	10	Dark blood in lumen of each tract in ovarian half only. Embryos dead in that region, but an embryo in vaginal end of uterus found with beating heart.
36	P/2/2/2/2/2/—/A	8	Abortion sign 48 hrs. after last injection. 10 dead implants. Deciduae capsularis yellowish brown. Tract vascularity reduced especially along mesometrium.
30	P/2/2/2/2/—/—/—/—/—/A	10	Loose elongated brown deciduae shifted in positions by strong contractions of swollen estrous uterus. *Ovulation:* 9 ova with granulosa cells in ampullae.
32	Same as No. 30	10	2 small decidual remnants flushed from right uterus. Oviducts active with 10 single-celled *ova* in 2nd–4th loops.
37	P/2/2/2/2/2/2/—/—/—/—/—/A	10	Abortion sign 48 hrs. after last injection. Deciduae gone at autopsy.
34	P/2 mg. 8 days/—/—/A	12	Much vaginal blood 50 hrs. after last injection. 4 dead brown elongated implants in each tract (3×8 mm). Two deciduae partially extruded through cervix into vagina.
35	Same as No. 34	12	Abortion sign 60 hrs. after last injection. Deciduae capsularis yellowish, flaccid, and easily torn. No embryos found within amniotic cavities.

[1] A.V.P. = after vaginal plug.
[2] P = day of finding vaginal plug of mating.
[3] A = autopsy.

thin endometrium. Adjacent to both edges of this band is an area of rugose mucosa, immediately beneath which lies the vascular intermediate zone of the decidua, which is the source of color in the pink stripes described above. Blood, escaping from the sinuses in this vascular area at about 9 days, accumulates in antimesometrial sinuses and deepens in color. These blood sinuses are most distinct just before the reopening of the antimesometrial lumen and the escape of blood into the vagina.

After the uterine lumen is reestablished, usually on the eleventh day of pregnancy, the light band becomes less apparent. This is due to changes in the thickness and the

nature of the endometrium in the former area of attachment and the pink areas which border it. After the twelfth day, the antimesometrial surface is relatively pale, while the metrial portion of each bead exhibits a mottled vascularity from capillaries forming in the enlarging placenta.

RESULTS

This investigation definitely supports the earlier observation that, once the injections of DCA are begun, the normal progress of pregnancy becomes dependent upon these daily injections. There were, however, some variations in the results within the different series. This could be expected in such animals having a large reservoir of subcutaneous oil after a series of injections, because the rate of absorption of DCA from these reservoirs was probably not uniform.

1. *The effect of daily injections of DCA on the progress of early pregnancy*

It was shown in the earlier report that implantation of the embryos was prevented if the injections were begun the day of mating. In the present investigation, however, it was found that if the injections were delayed until one or more days after the animals had mated, implantation and growth of the embryos proceeded normally as long as the DCA was given regularly. Mice 42 and 43 (Table 1, Part A) are typical of all those which were examined while receiving DCA daily. Their embryos were growing normally, and the embryonic hearts were beating on the tenth day of pregnancy. The DCA, therefore, did not have a deleterious effect upon the progress of early pregnancy.

2. *The effect on early pregnancy of withholding the daily injections of DCA*

The mice in this series were injected four or five days and then examined 48 or more hours after the last injection. Part B of the table shows a few of the many animals used. The results in this group differed radically from those in the preceding series. The embryos were usually dead and undergoing maceration. After four or five days, the uterus was partly or entirely cleared of the products of conception.

The appearance of blood in the vagina 42 to 60 hours after the last injection, was a reasonably sure sign that the pregnancy had been terminated. This blood must not be confused with that of the placental sign.

a. *Visible changes occurring in the vagina and uterus.* The amount of vaginal blood in the abortion sign varies. If the posterior deciduae occlude the uterine lumen until the blood in the more anterior portion has disappeared, little blood will be found in the vagina. When, on the other hand, all the deciduae degenerate at the same time, a copious amount of blood may be seen. So much blood was lost by one mouse that the sawdust in the cage was spotted.

The uteri of normal pregnant mice differ markedly from those of animals considered in this series. On the ninth day of pregnancy, the normal pregnant uterus has prominent blood vessels imbedded in the musculature. Blood, cherry-red in color, is normally seen in these vessels, in the lumen between the deciduae, and in the circular sinuses from the ninth day of pregnancy until after the placental sign.

This picture is duplicated in those animals regularly receiving DCA up to the time of autopsy (Table 1, Part A), but is radically changed after the hormone is withheld (Table 1, Part B). Approximately 48 hours after the last injection, and often before the abortion sign, the first difference noted in a superficial inspection of the uterus is that it has lost its bright red vascularity, and now is almost purple, and dark blood fills the lumen. On closer inspection, the prominent mesometrial blood vessels appear to end either at the uterine wall or soon after entering it, giving a definite impression of circulatory failure. Such blood vessels that do remain in the uterine walls are no longer continuous, but exist in broken patches. By the third or fourth day, the endometrium has become definitely ischemic, and the brownish deciduae may often be seen through the uterine walls.

As the embryos continue to degenerate, the uterus loses its sharply beaded appearance, is reduced in diameter, and becomes brownish in color. Several times we failed to note the presence of elongated embryos in the uterus until they were seen by transmitted light or were flushed from the uterus. When, in one animal, the autopsy was delayed until the approach of another estrous period, loosened embryonic remnants were being shifted back and forth in the reactivated uterus.

b. *Deleterious effects on the embryonic tissues.* The deciduae may appear entirely normal in the early stages of degeneration, but whenever the embryos are examined within their amniotic cavities, they are ischemic and flaccid, and tear easily. A few hours later, it is often impossible to find anything within the amnion that resembles the embryo. This contrasts sharply with normal foetal tissues, which, in spite of their thinness, are resilient and are not easily torn by the forceps.

After the embryo disintegrates within the amnion, changes in the external appearance of the deciduae are definite and conspicuous, but these changes depend upon the age of the embryo and upon the elapsed time between the last injection and the autopsy. A few observations of these characteristic color changes, never seen in normal deciduae, will clarify this point. Mouse 36 (Table 1, Part B), injected until the sixth day of pregnancy, was sacrificed two days later. The deciduae were generally spheroidal and the mesometrial (basal) portion of each was conspicuously and uniformly deep red, but the antimesometrial region was grayish-brown. A yellow band separated these two areas. No conspicuous vascularity ap-

peared in the blood-sinus areas because these sinuses are not normally developed at this age.

Mouse 35 was injected until the ninth day of pregnancy, by which time the blood sinuses are normally present in the deciduae. When examined three days after the last injection, the basal portion of the deciduae were generally pink except for the two deep red vascular areas in the regions of the newly formed blood sinuses. Each decidua capsularis was yellowish-brown and soft, even mushy, in texture. The deciduae were not spheroidal as in mouse 36, but were reduced in height and had begun to elongate in the axis of the uterine lumen, and were connected to the uterus by a slender stalk. No embryos were found in the amniotic cavities.

In another animal (mouse 30), in which autopsy was delayed for five days after the last injection, the deciduae were all gray or brown, spindle-shaped, loose in the uterine lumen, and in advanced stages of degeneration.

These macerating remnants remain in the uterus for varying lengths of time, but usually they are loose by the fourth day after the last injection and disappear by the sixth day. It was thought, at first, that complete maceration took place in the uterus, but several times we recovered one or more remnants from the vagina. In one mouse, an elongated brown remnant with its stalk still attached to the mesometrial wall of the uterus, was found extended through the cervix into the vagina. It would seem, then, that the disappearance of these embryonic remnants from the uterus depends both upon the time of survival of the deciduae and upon the increased uterine activity associated with returning estrus.

Deleterious effects do not necessarily occur simultaneously in embryos of the same animal. The conditions found in mouse 19 (Table 1, Part B) illustrate this point. The embryos in the ovarian ends of the uterine horns were dead, and the lumen between these embryos were filled with dark red blood. The vaginal halves of the horns, however, had embryos with beating hearts. The deciduae surrounding these living embryos occluded the posterior uterine lumen so that the blood associated with the more anterior dead embryos could not pass into the vagina.

This progressive deterioration was also shown in several other mice in which the tissues of posterior embryos were resilient.

c. *DCA and the corpora lutea of pregnancy.* In our first report, we thought that this hormone caused the corpora lutea to blanch and degenerate. This is not the case. We have now had the opportunity to examine the ovaries of a large number of animals which were killed while a series of injections was in progress. The corpora lutea remained large and vascular, and appeared to regress only after the death of the embryos.

Two possible explanations for the death of the embryos are tentatively proposed; either the DCA had directly or indirectly depressed luteal func-

tioning, or the embryos had become dependent on a higher titre of this injected progresterone-like hormone.

An interesting exception was shown in one of the animals (mouse 39, Table 1, Part A) in which the corpora lutea were reduced to small corpora albicantia. The embryos were alive, but had the appearance of being only 7 days old when they should have reached the11-day stage. It is quite possible that the DCA, begun the fifth day of pregnancy, substituted for the missing progesterone (Robson, 1939). Although nidation may have been late, the DCA possibly kept the pregnancy from being terminated.

d. *Ovulation after withholding DCA.* These observations were made to determine whether the injected hormone would inhibit ovulation for any considerable period after a series of injections. In one animal, an unfertilized fragmented egg was found in the oviduct approximately $7\frac{1}{2}$ days after the last injection. Its position in the isthmus indicates that the ovulation probably occurred two days earlier, i.e., on the fifth day after the last injection. Another mouse had eggs with granulosa cells in the ampullae on the fourth day, and similar ova were found in mice 30 and 32 on the fifth days after the last injection. The DCA, as used in these experiments, does not, therefore, inhibit ovulation normally following the emptying of the uterus.

3. *The effect of DCA on non-pregnant metestrous mice*

A few non-pregnant mice were injected with DCA for several days beginning at metestrous, which would approximate the time after ovulation that the pregnant animals received their first injection. These animals were carefully checked at two-hour intervals, beginning 30 hours after the last injection. No vaginal blood was found. The blood, therefore, that was associated with the abortion sign, was not a direct result of injected DCA.

4. *The effect of sesame oil on early pregnancy*

Another series of animals was injected daily with 0.2 cc. to 0.4 cc. of sesame oil to determine whether this vehicle for DCA adversely affected the progress of early pregnancy. No deleterious effects were observed. The subcutaneous reservoir of oil was still present after parturition, and caused adhesions and abnormalities in local tissues.

SUMMARY

This report is concerned with some of the effects of DCA on the progress of early pregnancy in the albino mouse. Daily subcutaneous injections for 4 to 6 days were started later than the day of mating so as not to interfere with embryo implantation.

Neither DCA nor the sesame oil interfered with the progress of early

pregnancy. Abortions usually occurred, however, if the injections were stopped before the eighth day of pregnancy. Blood in the vagina, the abortion sign, often found 42 to 60 hours after the last injection, indicated that the pregnancy was terminated. Inspection of the uterus showed color changes indicative of circulatory failure. Foetal tissues became flaccid, and degenerated. The normally spherical deciduae underwent radical color changes, elongated in the axis of the uterine lumen, and finally separated from the uterus as macerated remnants.

The corpora lutea of pregnancy usually retained their vascularity, and their degeneration was associated with the death of embryos. It is not known whether pregnancy was terminated because DCA had depressed luteal function, or whether the embryos had become dependent upon a higher titre of this progesterone-like hormone.

Apparently these injections did not harm the ovary. Ovulation occurred in several mice 4 to 7 days after the last injection.

Non-pregnant metestrous mice did not show vaginal blood after a series of injections of DCA.

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EFFECT OF THYROIDECTOMY ON HYPERTENSION, NEPHROSCLEROSIS AND CARDIAC LESIONS PRODUCED BY DESOXYCORTICOSTERONE ACETATE (DCA) TREATMENT IN THE RAT[1,2]

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IT WAS shown by Selye, Hall and Rowley (1943) that treatment with desoxycorticosterone acetate (DCA) of unilaterally nephrectomized rats given a high sodium intake resulted in a syndrome of which the main characteristics were hypertension, polyuria, nephrosclerosis, cardiac nodules and periarteritis nodosa. Some of these changes are so similar to those of hypertension in man that the assumption was made (Selye, Hall and Rowley, 1943) that mineralo-corticoids could be involved in human hypertension. This hypothesis has received additional support from recent work on the so-called meta-corticoid hypertension (Friedman and Friedman, 1949, 1951; Prado, 1950; Green, Saunders, Wahlgren and Craig, 1952; Salgado, 1953).

A number of measures have been reported to inhibit the development of the syndrome induced by DCA; we have found that hypophysectomy before or even during the hormonal treatment can inhibit some of the toxic manifestations of DCA overdosage (Salgado and Selye, 1952; Salgado and Selye, 1953). Green *et al.* (1952) independently have made similar observations and have shown in addition that the extirpation of the hypophysis is beneficial even in the stage of meta-corticoid hypertension.

We have not yet been able to identify the hypophyseal factor or factors that condition the body for the initiation and maintenance of the DCA overdosage syndrome in hypophysectomized animals. In this paper, our attention has been focussed on the possible role of the thyroid gland in this syndrome.

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[2] Part of this work was presented before the Société de Biologie de Montréal and also before the XIX International Physiological Congress in Montreal.

It was already known (Selye, Stone, Nielsen and Leblond, 1945) that thyroxine aggravates the manifestations of DCA overdosage. Hence, we studied the effect of thyroidectomy immediately prior to or during the administration of DCA. In one experiment, the effect of prophylthiouracil administration was also studied.

MATERIALS AND METHODS

Experiment No. 1.

Forty male Sprague-Dawley rats, weighing 50–65 gm., were divided into four groups as follows: 7 rats served as controls (Group I); 11 were treated with DCA (Group II); 12 were thyroidectomized and treated with DCA (Group III) and 10 received prophylthiouracil and DCA (Group IV). The animals were observed for 60 days.

Experiment No. 2.

Sixty-five male Sprague-Dawley rats, weighing 50–65 gm., were used: 30 served as controls and 35 were treated with DCA. After 4 weeks, 30 controls and 30 DCA treated animals were still alive. All the animals were then divided into groups of 10 animals each, as follows: *controls*—Group I, sacrificed; Group II, no treatment; Group III, thyroidectomized. *DCA treated*—Group IV, sacrificed; Group V, no other treatment; Group VI, thyroidectomized. The animals were observed for an additional 18 days period and were then sacrificed.

Experiment No. 3.

Forty-eight male Sprague-Dawley rats, weighing 50–65 gm., were treated with DCA After 39 days, 40 animals were still alive. Ten were then killed (Group I) and 15 were. thyroidectomized (Group III). These latter animals, together with the 15 remaining (Group II), were observed for another 23 days and then sacrificed.

All rats were unilaterally nephrectomized and given 1% NaCl to drink ad libitum. The diet consisted of a commercial chow (Purina Fox Chow). The treatment with DCA[3] consisted of one 20 mg. pellet implanted subcutaneously during each of the first 3 weeks of the experiment. Prophylthiouracil[4] was mixed with the food in a concentration of 0.1%. The body weight was measured weekly and the fluid intake (i.e., saline consumption) of each rat was measured daily. Blood pressure determinations were made by the microphonic method (Friedman and Freed, 1949) under light ether anesthesia, at the intervals indicated in the figures. The completeness of thyroidectomy was verified by inspection at autopsy and by such criteria as body and hypophyseal weights and by histologic studies of the pituitary gland. Data obtained from incompletely operated animals were omitted. At the conclusion of each experiment, the rats were killed and the organs rapidly removed. After fixation in Susa's fluid for 48 hours, these were dissected, weighed, sectioned at 6 μ and stained with hematoxylin and eosin or Mallory-Heidenhain for microscopic examination. To evaluate the pathologic changes objectively, the sections were read before verifying their identity and graded as follows: 0 = no lesions; 1+ = slight but definite lesions; 2+ = intermediate lesions; 3+ = maximal lesions.

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[3] We are indebted to Doctor E. Henderson of the Schering Corporation for the generous supply of DCA pellets.

[4] Prophylthiouracil was kindly provided by Charles E. Frosst and Co., Montreal.

RESULTS

Experiment No. 1.—During the course of this experiment, 1 rat died in Group I, 9 in Group II and 3 in each of Groups III and IV. The results of the blood pressure determinations, and the measurements of body weight and fluid intake are graphically presented in Figure 1.

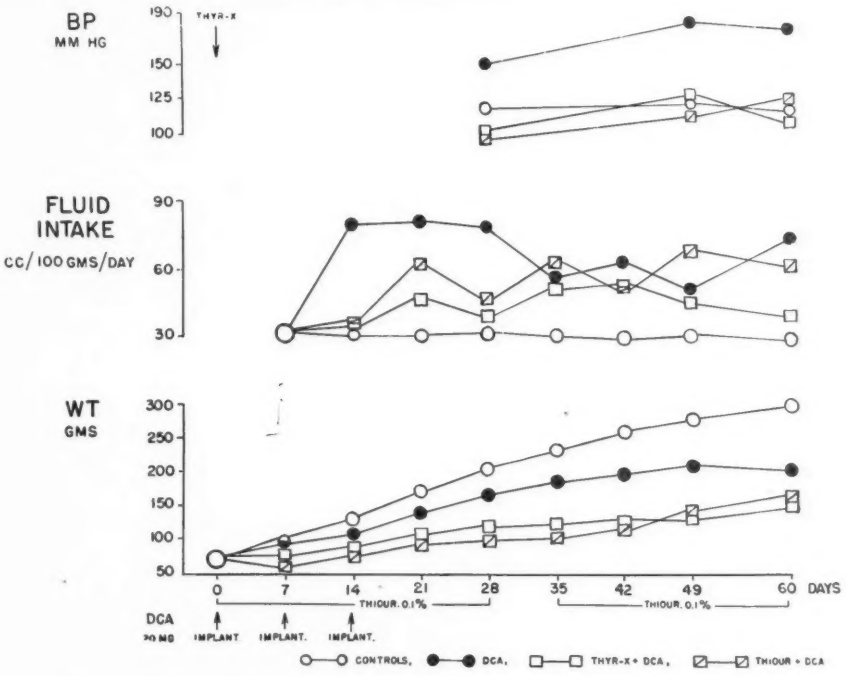

FIG. 1. Changes in body weight, blood pressure and fluid (NaCl 1%) intake of the rats in Experiment no. 1.

It can be seen that thyroidectomized or prophylthiouracil and DCA treated animals gained weight less than either absolute controls or DCA treated animals. The fluid intake of Group II increased, at the beginning of the experiment, to a higher level than the other groups. Later, the differences were not very striking. No elevation in blood pressure was detected in either Groups I (absolute controls), II (thyroidectomy and DCA) or IV (prophylthiouracil and DCA) while a very high and significant ($P < 0.01$) hypertension occurred in those receiving DCA.

The organ weights presented in Table 1 show that the heart and kidneys of all animals receiving DCA (Groups II, III and IV) were significantly larger ($P < 0.01$) than those of the controls (Group I). However, the heart

TABLE 1. THE ORGAN WEIGHTS AND QUANTITATIVE EVALUATION OF PATHOLOGIC LESIONS IN SALT TREATED, UNILATERALLY NEPHRECTOMIZED MALE RATS THYROIDECTOMIZED OR PROPHYLTHIOURACIL FED AND TREATED WITH DCA

Group and treatment	No. of rats	Organ weights in mg./100 gm. body weight		Pathologic lesions*					
				Nephrosclerosis		Cardiac lesions		Periarteritis	
		Heart	Kidney	Mean	No. with grade 2 or higher	Mean	No. with grade 2 or higher	Mean	No. with grade 2 or higher
I Control	6	304 ± 8	566 ± 16	0	0	0	0	0	0
II DCA	2	596 ± 60	1314 ± 111	2.6	9	2.5	9	1	3
III Thyroidectomy + DCA	9	406 ± 15	644 ± 16	0.2	0	0.7	0	1.5	5
IV Prophylthiouracil + DCA	7	446 ± 17	838 ± 21	0.3	0	0.3	0	0.7	0

All weights accompanied by the standard error of the mean.
* In the evaluation are included 7 animals of Group II which died before the end of the experiment.

and kidneys of the animals of Group II (receiving DCA alone) were significantly heavier ($P < 0.01$) than in Groups III and IV (thyroidectomy or prophylthiouracil and DCA). The kidneys showed a greater increase in weight in Group IV than in Group III ($P < 0.01$).

Histologic examination showed no abnormality in heart, kidneys or mesenteric and pancreatic vessels in Group I (controls). In Group II, the animals surviving or dead (the latter are also included in Table I) presented severe lesions of nephrosclerosis, myocarditis and periarteritis nodosa (see Plate 1).

In only three animals of Group III (thyroidectomy and DCA treated) a very slight basophilia in the renal tubules and an occasional hyalin cast

→

EXPLANATION OF PLATE 1

FIG. 1. Kidney of a DCA treated animal showing intense nephrosclerotic lesions. Hematoxylin and eosin (low magnification).

FIG. 2. Kidney of a similarly treated animal, but thyroidectomized at the beginning of the DCA treatment. See almost normal renal structure. Hematoxylin and eosin (low magnification).

FIG. 3. Periarteritic lesions in a pancreatic artery of a DCA treated animal. Mallory-Heidenhain (low magnification).

FIG. 4. Periarteritic lesions in a pancreatic artery of a similarly treated animal, but thyroidectomized at the beginning of the treatment. Mallory-Heidenhain (low magnification).

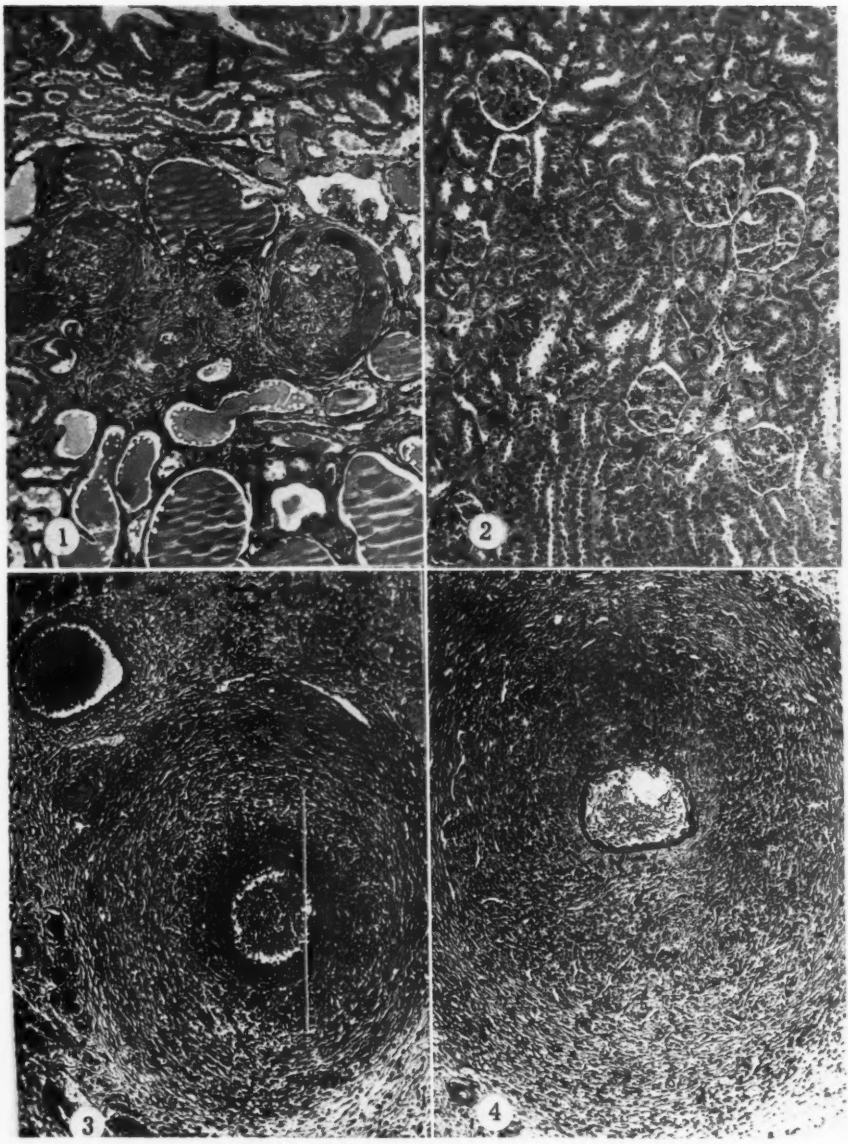

PLATE I

were seen. Only one animal had cardiac lesions and most of them showed extensive lesions of periarteritis nodosa in mesenteric and pancreatic vessels, these being of the same quality as those seen in Group II (see Plate 1); their extention, however, was greater. In the animals of Group IV (prophylthiouracil and DCA), the pathologic lesions were in general identical with those of Group III, except for periarteritic lesions which were absent in all the animals but one. The survival rate was increased both by thyroidectomy and by prophylthiouracil.

Experiment No. 2.—The results of this experiment are presented in Figure 2 and Table 2 in a manner similar to that of Experiment no. 1. During the experiment, 5 animals died before the thyroidectomy, which is not unexpected during a long treatment with DCA. After the thyroidectomy and subsequent division into groups, 1 animal of Group III (thyroidectomy), 3 animals of Group V (DCA) and 4 of Group VI died.

The most important finding shown in Figure 2 is that thyroidectomy induced a pronounced fall in the already high blood pressure.

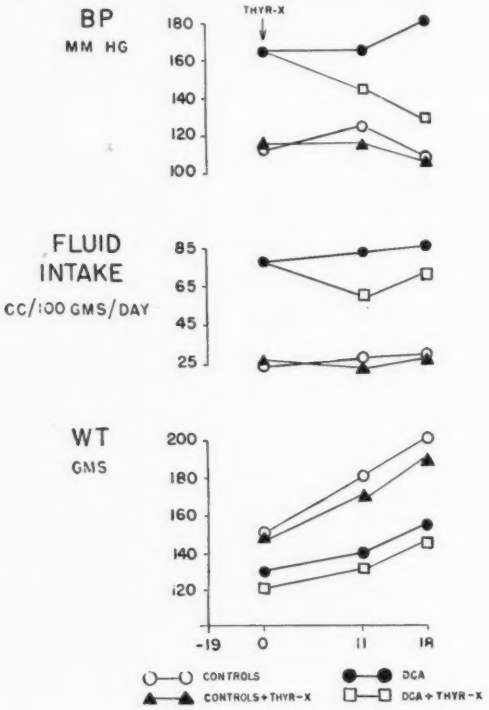

FIG. 2. Changes in body weight, blood pressure and fluid (NaCl 1%) intake in the rats of Experiment no. 2.

TABLE 2. THE ORGAN WEIGHTS AND QUANTITATIVE EVALUATION OF PATHOLOGIC LESIONS IN SALT TREATED, UNILATERALLY NEPHRECTOMIZED MALE RATS TREATED WITH DCA AND THYROIDECTOMIZED

Group and treatment	No. of rats	Organ weights in mg./100 gm. body weight		Pathologic lesions					
				Nephrosclerosis		Cardiac lesions		Periarteritis	
		Heart	Kidney	Mean	No. with grade 2 or higher	Mean	No. with grade 2 or higher	Mean	No. with grade 2 or higher
I Control (28 days)	10	320 ± 12	896 ± 26	0	0	0	0	0	0
II Control (46 days)	10	328 ± 8	730 ± 41	0	0	0	0	0	0
III Thyroid-ectomy	9	273 ± 13	573 ± 18	0	0	0	0	0	0
IV DCA (28 days)	10	502 ± 31	1288 ± 23	1	4	0.8	4	0.3	0
V DCA (46 days)	7	579 ± 31	1348 ± 104	2.8	7	1.8	5	1.5	2
VI DCA +Thyroid-ectomy	6	471 ± 21	928 ± 50	1.1	2	1	3	3	6

All weights accompanied by the standard error of the mean.

As Table 2 indicates, there is no difference between the weights of heart and kidney in Groups IV and V; hence, a longer treatment with DCA is not followed by an increase in the weights of these organs. Thyroidectomy did not completely inhibit the hypertrophy of kidney and heart, since these organs were larger in Group VI than in Groups II and III ($P < 0.01$). However, there is still a significant difference ($P < 0.01$) between the weights of kidneys, but not the weights of hearts, in Group VI (thyroidectomy) compared with those of Groups IV and V. Histologic examination showed that thyroidectomy arrested the development of renal and cardiac lesions and enhanced the development of the periarteritis nodosa.

Experiment No. 3.—This experiment was designed to determine the effect of thyroidectomy on a syndrome induced by a more prolonged treatment with DCA. During the experiment, 9 animals died before the thyroidectomy and afterwards 8 animals died in Group II (DCA) and 1 in Group III (DCA and thyroidectomy).

As seen in Figure 3, there is only a transitory fall in blood pressure following thyroidectomy. Except for this and the increased severity of pathologic lesions at the moment of thyroidectomy, the results are essentially

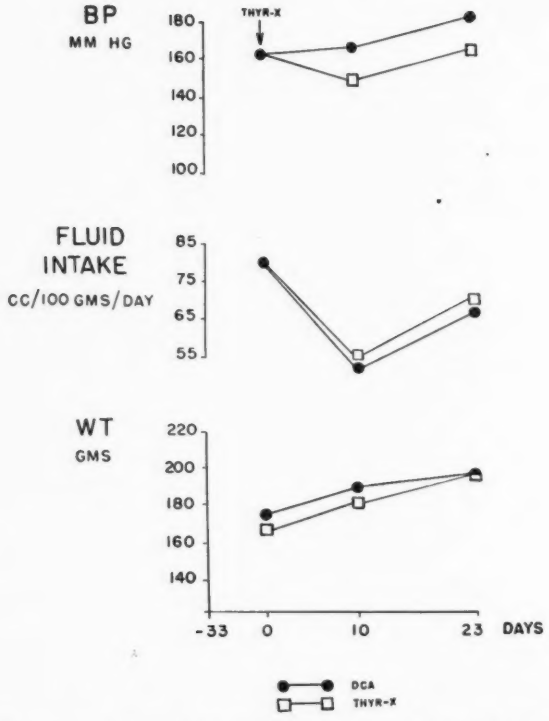

FIG. 3. Changes in body weight, blood pressure and fluid (NaCl 1%) intake of the rats in experiment no. 3.

TABLE 3. THE ORGAN WEIGHTS AND QUANTITATIVE EVALUATION OF PATHOLOGIC LESIONS IN SALT TREATED, UNILATERALLY NEPHRECTOMIZED AND THYROIDECTOMIZED MALE RATS TREATED WITH DCA

Group and treatment	No. of rats	Organ weights in mg./100 gm. body weight		Pathologic lesions					
				Nephrosclerosis		Cardiac lesions		Periarteritis	
		Heart	Kidney	Mean	No. with grade 2 or higher	Mean	No. with grade 2 or higher	Mean	No. with grade 2 or higher
I DCA (39 days)	10	406 ± 13	1317 ± 137	1.5	4	1.7	4	0.5	0
II DCA (62 days)	7	414 ± 19	1436 ± 123	2.5	6	2.1	5	1.0	3
III DCA +Thyroid-ectomy	14	390 ± 13	984 ± 58	1.5	6	1.7	8	2.2	8

All weights accompanied by the standard error of the mean.

the same as in the preceding experiment. In this experiment again the survival rate was clearly increased by thyroidectomy.

DISCUSSION

It appears from our results that thyroidectomy can inhibit the development of hypertension, nephrosclerosis and cardiac nodules induced by DCA overdosage. On the contrary, periarteritis nodosa is actually aggravated in the absence of the thyroid gland. This means that periarteritis nodosa in the rat is not a consequence of hypertension only. The prophlogistic activity of DCA described by Selye (1946) could well be responsible for the development of such lesions, this activity being enhanced by the absence of the thyroid gland.

To explain the fact that the fall in blood-pressure in our Experiment 3 was not very marked, two possibilities have to be considered: 1) that an irreversible period of hypertension had been reached; 2) that the periarteritic lesions observed caused a secondary rise in blood pressure. The second possibility appears to be the more likely because, according to our preliminary experiments, thyroidectomy followed by cortisone administration seems to prevent periarteritis nodosa and in this case even secondary hypertension, cardiac and renal lesions, do not develop.

It is interesting to note that periarteritis nodosa is observed in thyroidectomized and not in thiouracil treated animals receiving DCA, although in both instances an increased secretion of thyrotrophic hormone exists. More experiments will be necessary to clarify this point. However, attention must be drawn to the fact that the diminution of thyroid function could have played a role in some cases of periarteritis nodosa which have been reported in human beings and animals after the administration of sulfa drugs (Rich, 1942; French, 1946; Lichtenstein, 1946; More, McMillan and Duff, 1946; Thompson and Zeek, 1945), iodine (Rich, 1946), and thiouracil (Marine and Baumann, 1945; Gibson, 1945).

SUMMARY

The effects of thyroid extirpation and prophylthiouracil administration were studied in male rats drinking a 1% solution of NaCl, which were unilaterally nephrectomized and DCA implanted. Thyroidectomy performed simultaneously with the beginning of DCA treatment inhibited hypertension, and renal and cardiac lesions. It enhanced the periarteritis nodosa. Cardiac and renal enlargement was diminished but present. Mortality was diminished. Thiouracil administration, when started simultaneously with DCA, produced effects similar to those of thyroidectomy, except that no periarteritic lesions were present. Thyroidectomy performed during DCA administration arrested the progress of renal and cardiac lesions and en-

hanced the periarteritis nodosa. It markedly influenced the hypertension, and it diminished renal and cardiac enlargement. Mortality was diminished. The significance of these findings, as well as the positive conditioning role of thyroidectomy in the pathogenesis of periarteritis nodosa, has been briefly discussed.

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STUDIES OF ACTH ACTION UPON PERFUSED BOVINE ADRENALS: CORTICOSTEROID BIOSYNTHESIS IN ISOLATED GLANDS MAXIMALLY STIMULATED WITH ACTH[1]

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INTRODUCTION

THE ability of ACTH to stimulate corticosteroidogenesis under *in vitro* conditions was first demonstrated in the isolated cow gland perfused with homologous citrated blood (Hechter, 1949). This effect of ACTH was subsequently achieved in simpler systems by incubation of rat (Saffran *et al.*, 1952; Hofmann and Davison, 1953; Birmingham *et al.*, 1953) and cow (Haynes *et al.*, 1952) adrenal slices, in Ringers solution. The *in vitro* action of ACTH on the perfused adrenal was initially obtained in arterially cannulated glands. Later it was found that higher rates of corticosteroid biosynthesis could be achieved by lacerating the surface of the gland and perfusing the adrenal via the adrenal vein. The venous perfused-lacerated bovine gland (V-L gland) was later utilized as a biochemical tool to characterize the corticosteroids formed under the influence of added ACTH and to evaluate the metabolic pathways involved in corticosteroid biosynthesis. These results have been previously reviewed (Hechter *et al.*, 1951; Hechter, 1951a; Pincus, Hechter and Zaffaroni, 1951; Hechter, 1953).

Having available an *in vitro* system for the study of the corticosteroidogenic activity of ACTH in an intact adrenal gland, studies were undertaken to test in direct fashion, various aspects of ACTH action. In this first paper of a series dealing with ACTH, the rate of corticosteroid biosynthesis achieved by arterially perfused glands is compared to that of venous perfused-lacerated glands, in the absence of added ACTH and in the presence

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of concentrations of ACTH which induce maximal stimulation. In addition the influence of certain factors which affect the rate of corticosteroid biosynthesis in glands stimulated maximally with ACTH will be presented.

METHODS

Bovine glands, obtained at the local slaughter house, were transported to the laboratory in cold 0.9% NaCl solution. They were prepared for perfusion (either arterial, or via the adrenal vein) using methods previously described (Hechter *et al.*, 1953). The glands were perfused at 38° C. with homologous citrated blood, unless otherwise stated. The perfusion apparatus employed (Hechter, 1948) and the preparation of blood and other media for perfusion (using rat liver as a biological filter) have been described in detail (Hechter *et al.*, 1953).

Evaluation of corticosteroid output from perfused glands was accomplished by chemical analysis of the corticosteroid content of the perfusion medium, before and after it had circulated through the adrenal. Corticosteroids in adrenal perfusates were extracted by procedures to be described and measured as formaldehydogenic substance (F.S.) following periodate oxidation using a modification of the procedure of Daughaday *et al.* (1948) (11-desoxycorticosterone as standard).

The ACTH used was kindly supplied by the Armour Co. The first experiments (performed in 1948–49) involving arterial perfusion of cow glands employed a crude ACTH preparation relative to the highly purified ACTH fractions subsequently developed. The later studies (carried out during 1950–52), involving perfusion of the venous cannulated gland, employed the more highly purified preparations then available. All ACTH samples were used immediately after solution of the dry powder in physiological saline. All doses of ACTH employed in these experiments were sufficiently large so that the concentration of ACTH did not act as a limiting factor in the rate of corticosteroid biosynthesis. In these experiments, the concentrations employed ranged from 1 to 43 I.U. per liter. As the next paper in this series will demonstrate, the minimal concentration of ACTH necessary to evoke a maximal response in the perfused bovine adrenal is in the range of 0.1 I.U. per liter (Macchi and Hechter, 1954).

Extraction of corticosteroids from adrenal perfusates

The development of methods for extracting corticosteroids from adrenal perfusates in this laboratory has been discussed previously (Hechter, 1951b) and reviewed in detail (Levy and Kushinsky, 1954). It was observed that while acetone extraction and direct $CHCl_3$ extraction removed corticosteroid from perfusates (as determined both by F.S. and glycogenic assays), a large amount of non-corticoid F.S. material was simultaneously extracted. This complicating F.S. material could be removed only by extensive fractionations. Later, it was discovered that corticosteroids in adrenal perfusates are dialyzable into saline while the bulk of the lipids and the non-corticoid F.S. remain within the dialysis sac. The corticosteroids could then be obtained by chloroform extraction of the dialysates of blood perfusates, without significant contamination by either lipoids or phosphatides. While other methods were available, the dialysis method was simplest and became the method of choice in these studies. After publication of our initial work (Hechter, 1949), others have subsequently employed dialysis as a procedure for extraction of corticosteroids from blood (Zaffaroni, 1953).

The results to be reported were obtained with the following method of dialysis: One volume of blood or plasma perfusate was dialyzed against 10 volumes of 0.9% NaCl solu-

tion in a rocking apparatus for 60 hours in the refrigerator at 2–5° C. The dialysate thus obtained was extracted with 0.5 volume of $CHCl_3$ three times. The chloroform extracts were washed with water (0.1 vol ×3), dried with anhydrous sodium sulfate, and then distilled to dryness *in vacuo* under nitrogen. The residues thus obtained contain only trace amounts of lipids and non-corticoid F.S. Corticosteroid estimation was carried out directly on aliquots of these residues. Generally, duplicate 20 ml. aliquots of perfusates were dialyzed to obtain estimates of the corticosteroid content. When lower values were expected (e.g., in the control non-ACTH samples), larger aliquots ranging from 40–50 ml. were employed.

To check the efficiency of the dialysis procedure used, cortisone was tested as a model corticosteroid. Table 1 illustrates the recovery of this steroid added to blood in the range of concentrations found in these studies to occur in adrenal perfusates. It will be seen that at the lowest concentration tested (200 μg. cortisone per 100 ml.) only 45% is recovered. With increase in cortisone concentration, the recovery increases to a maximal value of 88% at concentrations of 1000 μg. per 100 ml. If corticosteroids were freely diffusible, only 91% recovery of added cortisone is theoretically possible by this dialysis procedure. These data, therefore, demonstrate that a model corticosteroid added to blood is satisfactorily removed by dialysis in the concentration range from 500 to 1000 μg. per 100 ml. blood. No explanation is available to account for the lower degree of recovery at 200 μg.%. It should be emphasized that the per cent recovery of the naturally occurring mixture of corticosteroids (primarily hydrocortisone and corticosterone) may not be strictly comparable to the results obtained with cortisone. A second period of dialysis of adrenal perfusates did not yield additional amounts of corticosteroid after the first dialysis period. This is also the case following tryptic digestion of dialyzed adrenal perfusates; this suggests that the corticosteroids in adrenal perfusates are not present as a protein-complex so firmly bound that they cannot be removed by simple dialysis. Subsequently, when corticosterone and hydrocortisone were available in sufficient

TABLE 1. COMPARISON OF THE RECOVERY OF VARIOUS CONCENTRATIONS OF CORTISONE FROM 20 ML. OF BOVINE BLOOD BY THE DIALYSIS EXTRACTION METHOD

Concentration added cortisone μg./100 ml.	Actual μg. cortisone in 20 ml. aliquot	Total μg. F.S. dialyzed from 20 ml. blood with added cortisone*		Net μg. F.S. representing recovery of added cortisone		Average net μg. F.S.	Mean per cent recovery
		Expt 1	Expt 2	Expt 1	Expt 2		
200	40	30 31	30 30	19 20	16 16	18	45
500	100	83 87	76 80	72 76	62 66	69	69
1000	200	183 194	183 189	172 183	169 175	175	88
10000	2000	1910 2120	1405 1455	1899 2109	1391 1441	1710	86
μg. F.S. recovered from 20 ml. bovine blood without added cortisone		11	14				

* Recovery values for each experiment were obtained by performing duplicate determinations for each concentration level of added cortisone.

quantities, it was shown that these steroids, and 11 desoxycorticosterone as well, were recovered to the extent of 80–95% from blood containing 1000 μg.%, by this dialysis method. However, recovery data at various lower levels of steroid concentration were not obtained.

Evaluation of data

The results to be presented throughout this paper have been calculated as μg. corticosteroid output per gram of adrenal tissue (wet weight) per hour of perfusion. This method of evaluating adrenal corticosteroidogenic response assumes certain relationships which are not necessarily present in our isolated adrenal preparations. The principal difficulty arises in that this method of expressing our results assumes that the entire adrenal tissue is uniformly perfused. This, however, is not necessarily the case. When adrenals, whether perfused arterially or through the vein, were subsequently circulated with a variety of dyes (including Chicago blue) at the termination of the experiment it was discovered that variable amounts of the adrenal tissue were unstained. This was particularly true with the arterially perfused glands and much less so in the V-L perfused glands. It is thus apparent that a certain fraction of adrenal tissue, difficult to quantitate, is ineffectively circulated during the course of these experiments. A second assumption involved is that the adrenal cortical tissue, the presumed site of corticosteroid biosynthesis, is regarded as directly proportional to the total adrenal weight. This appears to be a fair assumption since Macchi (unpublished work) has found that the percentage of medullary tissue present in cow glands varies from only 20.9 to 30.7% of the total gland weight, averaging 26%. Perhaps a more serious difficulty arises in that there may be variable fat content in the adrenal, which is not taken into account when results are evaluated on a total weight basis. Finally, when glands perfused for different time intervals are compared on this basis, it is assumed that the rate of corticosteroid output during the entire duration of the perfusion is constant. This however, is not necessarily the case, as will be shown in the second paper of this series (Macchi and Hechter, 1954).

RESULTS

(*A*) *Studies on arterially perfused glands*

Effect of ACTH upon glands perfused with blood

Cow glands obtained during the winter and spring of 1948–49 were perfused through the aorta with homologous citrated blood. The adrenal venous outflow was removed from the system and was not permitted to recirculate through the gland. The following experimental design was employed: a sample of the perfusion medium was removed from the apparatus prior to initiation of adrenal perfusion to obtain the initial F.S. content of the medium. Then pulsatile perfusion of the adrenal was initiated under known conditions of *mean* arterial pressure. Several successive samples of the adrenal effluent were collected to obtain information concerning the output of F.S. in the absence of added ACTH. After this, a single large dose of ACTH was injected into the perfusion reservoir, mixed, and additional adrenal venous samples obtained. Finally, a sample of the residual blood in the pump which had not been perfused through the gland was

taken as a second control. This sample was assayed to check the possibility that the F.S. content of blood might be altered as the result of continuous circulation through the pump (but not the gland) or directly by the added ACTH. The mean F.S. values for the blood before perfusion through the adrenal gland was 75 ± 3.12 (standard error) μg. per 100 ml.; at the end of the experiment the mean F.S. was 74 ± 3.13 (standard error) μg. per 100 ml: this finding demonstrates that the F.S. content of the blood containing ACTH was not altered significantly as a result of recirculation through the pump, if it did not pass through the gland.

The results obtained are shown in Table 2. It will be observed that:

(a) In the absence of added ACTH, isolated cow glands perfused with blood release only small amounts of corticosteroid. This occurs mainly dur-

TABLE 2. ARTERIAL PERFUSION OF COW GLANDS WITH WHOLE CITRATED BLOOD

Expt. No.	Gland wt. gm.	ACTH conc., I.U./liter	Sample No.	Interval of perfusion, min.	Blood flow, cc./min./gm.	Pressure, mm. Hg.	Uncorrected output, μg./gm./hr.
1	17.2	0	1	0–45	0.26	70	+3
			2	45–90	0.24	70	−2
		43	3	90–170	0.14	70	+38
2	17.7	0	1	0–45	0.12	65	+2
			2	45–115	0.08	75	0
		30	3	115–145	0.08	90	+16
3	26.4	0	1	0–17	0.22	20	+8
			2	17–32	0.25	20	−5
			3	32–48	0.24	20	−1
		40	4	48–65	0.21	20	+25
			5	65–85	0.19	20	+35
			6	85–111	0.15	20	+38
			7	111–144	0.11	20	+22
4	24.0	0	1	0–20	0.21	40	+23
			2	20–57	0.22	40	+10
			3	57–98	0.20	40	+ 1
		20	4	98–130	0.26	40	+105
			5	130–187	0.15	40	+ 83
			6	187–277	0.09	40	+ 72
			7	277–337	0.15	90	+105
5	21.2	0	1	0–32	0.17	120	+15
		18	2	32–125	0.11	130	+47
			3	125–190	0.12	140	+55
6	25.1	0	1	0–51	0.39	60	0
		11	2	51–120	0.28	60	+48
			3	120–207	0.23	60	+56
			4	207–240	0.60	60	+60

ing the early phase of perfusion and tends to fall off toward zero with increasing periods of perfusion. The average output in the absence of added ACTH is about 5 μg./gm./hr.

(b) In the presence of ACTH, the output of F.S. is uniformly increased. Although each experiment should be considered separately since the conditions of preparation and perfusion are somewhat different, it may be calculated that the average output of F.S. in the presence of ACTH is about 47 μg./gm./hr. It should be noted that the increased output induced by ACTH is maintained for several hours.

(c) The limited data available indicate that the effect of ACTH in different glands does not appear to be influenced markedly by differences in either mean arterial pressure or the rate of blood flow. It is striking to note in the light of current concepts of fluid exchange between capillaries and tissues, that ACTH still induces its effect upon isolated cow glands perfused at a mean arterial pressure of 20 mm. Hg.

(d) While the effect of ACTH occurs at a wide range of adrenal blood flows, it is nevertheless evident that the ACTH preparation employed does decrease the adrenal blood flow. Decreased blood flow, *per se*, does not increase corticosteroid output as has been shown by the fact that vasoconstriction induced by epinephrine *in vitro* does not stimulate corticosteroid output (Hechter *et al.*, 1951). In retrospect, the vasoconstrictor effects observed with crude ACTH preparations were probably due to posterior pituitary contamination. Using the more highly purified ACTH preparations available in recent years, we have been able to stimulate corticosteroid output from isolated cow adrenals without any significant diminution of adrenal blood flow. For example, the ACTH effects to be described upon the V-L perfused gland are achieved without any significant diminution of blood flow through the adrenal.

Effect of ACTH upon glands perfused with plasma or blood diluted with plasma

It was of interest to determine whether ACTH action could be obtained using a simpler perfusion medium than blood. Accordingly glands (weighing between 15.5 to 24 gms.) were perfused with ACTH using either plasma exclusively or with blood-plasma mixtures as the perfusion medium. Table 3 illustrates the results obtained. It will be seen that using 100 per cent plasma as the perfusion medium, the ACTH effect is no longer obtained consistently. In the four experiments of this series there is clear evidence of augmented corticosteroid release in only one case (Expt. 2); there is a possible ACTH effect in another (Expt. 4), but in the remaining experiments (Expts. 1 and 3) with plasma, ACTH had no significant effect. When 17–20% whole blood is added to plasma, it will be observed that a clear

TABLE 3. COMPARISON OF ACTH RESPONSE IN GLANDS PERFUSED WITH PLASMA AND PLASMA DILUTED WITH WHOLE BLOOD

Expt.	Medium plasma, %	Blood, %	ACTH conc., I.U./liter	Perfusion time	Blood flow, cc./min./g.	Blood pr., mm. Hg	Corticosteroid output, μg./gm./hr.
1	100	0	0 30	120 120	0.8 0.7	50 50	2 8
2	100	0	0 17	60 60	1.0 0.9	90 100	8 43
3	100	0	0 20	90 160	1.0 1.0	60 65	0 −2
4	100	0	0 30	31 82	1.0 0.5	50 50	12 17
5	83	17	0 10	60 80	0.30 0.15	70 70	1 40
6	80	20	0 23	70 80	0.25 0.16	40 40	6 28
7	80	20	0 40	20 20	0.5 0.5	100 100	3 48

ACTH effect was again obtained. As an incidental finding, it was observed that the adrenal blood flow was significantly higher when glands were perfused with plasma instead of blood or blood diluted with plasma. That ACTH action *per se* does not require the presence of erythrocytes in the system is shown by the finding that a single gland (Expt. 2, Table 3) perfused with plasma alone exhibited a normal ACTH effect. The *in vitro* studies involving rat (Saffran *et al.*, 1952) and beef (Haynes *et al.*, 1952) adrenal tissue incubated in simple Ringer's solution have established the fact that ACTH action upon corticosteroid biosynthesis does not depend upon the presence of erythrocytes. This, however, should not be taken to indicate that the presence or absence of erythrocytes is without influence upon the magnitude of the corticosteroid output achieved in the presence of ACTH. Nevertheless, the results shown in Table 3 suggest that effective ACTH stimulation of the isolated cow adrenal is more consistently obtained when the gland is perfused with erythrocytes in the medium.

Accordingly, in all further experiments described in this paper citrated blood was employed as the perfusion medium.

(B) *Studies on venous-cannulated-lacerated glands*

The technical advantage of the V-L perfused gland relative to the arterially perfused preparation have been previously described (Hechter *et al.*, 1953). Studies were undertaken to investigate the effect of ACTH upon the V-L preparation.

In these experiments the glands were first perfused for 30 minutes with blood (not containing added ACTH) in order to "warm-up" the previously chilled gland. As a control, the gland was then perfused with blood for 18–20 minutes in order to obtain the level of corticosteroid output in the absence of added ACTH. Thereafter, the adrenal was perfused for 60 to 72 minutes with blood containing ACTH freshly added at 18 minute intervals. As in the previously described experiments involving arterial perfusion, the adrenal effluent was removed from the system and did not recirculate through the gland. Perfusion was carried out under an arbitrary set of conditions where the non-pulsatile perfusion pressures were adjusted to permit a constant adrenal blood flow rate of 1 liter per hour, irrespective of gland weight. Glands from 32 cows, 6 steers and 2 bulls were studied during various periods of the year. The major portion of the experiments were carried out on glands obtained from October through May.

Cow Adrenals

Table 4 summarizes the results obtained with glands studied during various periods of the year. Here the results are presented as the increase in corticosteroid output over and above the control output in the absence of ACTH.

Examination of these data reveals that there is considerable variation from gland to gland within any season. The mean values for ACTH induced corticosteroid output obtained during the Autumn, Winter, and Spring are approximately similar in both magnitude and extent of variation. Statistical evaluation demonstrates that the mean ACTH effects observed during these seasons are not significantly different. This is not the situation during the summer months, where there appears to be diminished responsivity to ACTH. While it is inappropriate to attach physiological significance to only 3 experiments (given this range of individual variation), it can be calculated that the difference between the mean ACTH response obtained during the summer and all other seasons combined is statistically significant ($t = 2.28$; $p < 0.05 > 0.02$). In this connection it is important to

TABLE 4. SEASONAL INFLUENCE UPON THE TOTAL CORTICOSTEROID OUTPUT OF ISOLATED PERFUSED COW ADRENAL GLANDS MAXIMALLY STIMULATED WITH ACTH

Season	No. glands	Average net corticosteroid output,* μg./gm./hr.
Summer	3	121 ± 30.3†
Autumn	13	309 ± 14.7
Winter	9	352 ± 21.4
Spring	7	280 ± 20.0

* Corticosteroid output in excess of that obtained in the absence of exogenous ACTH.
† Standard error of the mean.

TABLE 5. TOTAL CORTICOSTEROID OUTPUT OF ISOLATED PERFUSED COW ADRENAL GLANDS IN THE ABSENCE AND PRESENCE OF ACTH

Preparation	ACTH dose	No. experiments	Average corticosteroid output, μg./gm./hr.	ACTH	Blood flow, ml./min./gm.	
				Non-ACTH	Average	Range
V-L Perfused	0	29	17 ± 6.55*	19	0.88	0.5–1.5
	Maximal	29	328 ± 36.2			
Arterial Perfused	0	6	4.6 ± 2.64	10	0.19	0.08–0.60
	Maximal	6	47 ± 10.3			

* Standard error of the mean.

note that Dr. Robert Haynes (private communication) working with cow adrenal slices at the Worcester Foundation, likewise found markedly diminished responsivity to ACTH during the summer of 1952. While this might suggest that the summer seasonal phenomenon observed may be real, subsequent work by C. A. Fish (private communication) at this institution has shown that perfused cow glands studied during the subsequent summer of 1953 did not exhibit this diminished response to ACTH. While the basis for our original observation is obscure, the conclusion appears merited that the findings obtained by us and Haynes were due to local conditions prevalent in the Worcester area in the summer of 1952 and do not appear to be general phenomenon related to seasonal variation.

The basis for the considerable individual variation observed throughout the year is not known. However, the variations encountered should not be surprising when it is considered that the glands and blood used for perfusion were obtained from animals of variable age, genetic strain and weight, some with minor bovine diseases, whose previous nutritional regimen was undoubtedly variable.

In Table 5, the results obtained with the V-L perfused cow gland are compared to those obtained with arterially perfused glands for comparable periods of the year (October to May). This excludes the results obtained during the summer months with the V-L preparation, since experiments with arterially perfused glands during the summer months were not available. Moreover, for the reasons cited above, the data on V-L glands studied during the summer of 1952 do not appear to conform to the general pattern. In general, it was observed in the individual gland perfused via the vein that the corticosteroid output in the presence of ACTH was proportional to that obtained in the absence of added ACTH. Thus, glands which exhibited poor response to ACTH, had a low initial rate of corticosteroid output in the absence of ACTH.

It will first be seen from Table 5 that the V-L perfused cow gland produces about 7 times more corticosteroid per unit weight per unit time than does the arterially perfused cow preparation in the presence of ACTH and about 4 times in the absence of added ACTH.

Since all other factors involved in the experimental situation are basically similar, the increased corticosteroid production and relatively larger ACTH response observed in V-L perfused glands must be related solely to the method of preparing glands for perfusion. A possible basis for this finding is indicated in Table 5. It will be seen that the V-L perfused gland, on the average, has a blood flow about 5 times greater than that observed with the arterially perfused gland. This suggests that an increased number of adrenocortical cells are more effectively perfused per unit time in the V-L preparation relative to the arterially perfused gland with consequent effects upon corticosteroidogenic capacity. While the superiority of the V-L gland in this respect may be explicable solely on this basis, it is not known whether additional factors are involved.

Relation of gland weight and adrenal blood flow to corticosteroid biosynthesis in V-L glands

The data presented in Table 5 are derived from cow glands which varied in weight from 10 to 28 grams. Since all of these isolated glands were perfused at a constant rate of blood flow per gland, the absolute rate of blood flow in terms of cc./min./gm. gland as well as the amount of adrenal tissue varied in these experiments. It was possible, therefore, to study the relationship between adrenal blood flow and adrenal weight upon corticosteroid output rates in the perfused gland. Figure 1 illustrates the data which

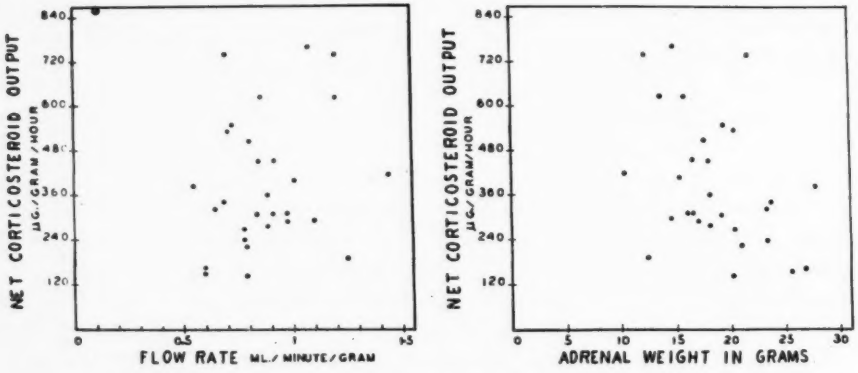

FIG. 1. The influence of unit blood flow and weight upon the rate of corticosteroid biosynthesis following maximal ACTH stimulation of cow adrenals.

TABLE 6. COMPARISON OF THE CORTICOSTEROID OUTPUT OF V-L PERFUSED ISOLATED STEER AND BULL ADRENALS IN THE PRESENCE OF A MAXIMAL EFFECTIVE DOSE OF ACTH

Type of bovine adrenal	No. experiments	Net corticosteroid output, μg./gm./hr.
Steer	1	20
	2	−7
	3	−5
	4	146
	5	146
	6	180
	Average	80
Bull	1	144
	2	198
	Average	171

show that there is no significant correlation at the 5% level of either adrenal blood flow in the range from 0.5 to 1.5 ml. per min. per gm. (correlation coefficient = 0.219) or adrenal weight in range from 10 to 28 grams (correlation coefficient = −0.309) upon the rate of corticosteroid biosynthesis of these ACTH stimulated cow glands.

Comparison of steer, bull and cow glands

Steer and bull glands obtained at slaughter during Autumn and Spring were perfused with ACTH under identical conditions as those employed previously with V-L cow glands. The results of these studies are shown in Table 6. The data demonstrate that of the 6 steer glands studied, 3 exhibited little or no response to ACTH, while the remaining 3 showed a response comparable to the low limits of the normal range in the V-L perfused cow gland (cf., Table 4). The differences between steer and cow glands are statistically significant ($P < .005$). The two experiments with bull glands yield intermediate values between the results obtained with cow and steer glands, but the limited data do not permit statistical evaluation. The suggestion from these studies is that sex and/or castration may influence the capacity for corticosteroid biosynthesis.

DISCUSSION

Recognizing the physiological limitations of the perfusion technic, which have been previously discussed (Hechter, 1951a), our primary objective has been to obtain an *in vitro* adrenal system wherein certain aspects of ACTH action upon corticosteroid biosynthesis might be studied in direct fashion. The results presented in this paper demonstrate that the isolated, perfused adrenal of the cow is a satisfactory system for demonstrating the corticosteroidogenic activity of ACTH. Cow adrenal glands respond to *in*

vitro ACTH by an increase in the rate of corticosteroid output, the magnitude of which is dependent upon the method of perfusion. Although marked effects of ACTH are obtainable in both types of preparations, the absolute rates of corticosteroid production are markedly different. Glands perfused via the aorta secrete at a rate only about $\frac{1}{7}$ of that obtained by perfusion of venous cannulated lacerated glands in the presence of ACTH and about $\frac{1}{4}$ in the absence of added ACTH. The superiority of the V-L perfused gland with respect to corticosteroidogenic capacity appears in all probability to be related to the more effective circulation of adrenocortical tissue achieved by this method of preparing glands for perfusion.

Comparison of V-L perfused cow and steer glands

In comparing the response of various types of bovine glands to concentrations of ACTH which evoke maximal stimulation, the finding was made that steer glands either fail or respond poorly to ACTH, relative to cow adrenals. While further work is necessary to establish these points definitively, one suggestion which emerges from these studies is that the capacity for corticosteroidogenesis in bovine adrenal glands may be influenced by sex differences and/or castration. Alternatively, lactation in cows may act as a stimulus to increase adrenocortical capacity to produce steroid. Whatever the basis for these differences between cow and steer glands, ultimately they appear to be a reflection of basic differences in the enzymatic systems involved in corticosteroid biosynthesis. In studies carried out in collaboration with Dr. David Stone (unpublished work), we have observed that steer adrenals perfused either with ACTH or progesterone (in the absence of ACTH) produce small amounts of corticosterone and little or no 17-hydroxycorticosterone as compared to the approximate 1:1 ratio of these two major corticosteroids occurring in the perfusates of cow adrenals perfused under identical conditions. These findings on the nature of the corticosteroids released from steer and cow glands suggest that the 17-hydroxylating enzyme system in adrenal tissue (necessary for the conversion of progesterone to 17-hydroxyprogesterone) is either inhibited or absent in the steer adrenal gland. Plager and Samuel (1953) likewise have found that the 17-hydroxylating enzyme activity demonstrable in cow adrenal homogenates is absent or markedly reduced in steer glands.

The implication from these findings is that steers may produce small amounts of corticosterone and little or no 17-hydroxycorticosterone relative to cows *in vivo* as well as *in vitro*. If, as is generally assumed, glucocorticoids like 17-hydroxycorticosterone and corticosterone are the principal products of the adrenocortical secretion involved in resistance to noxious agents, one might expect to find differences between steers and cows with respect to ability to withstand disease and other stress situations as-

sociated with their daily life. Such differences, however, are not known and the steer does not exhibit evidence of adrenal hypofunction. This may merely illustrate the limitation of an *in vitro* approach to the problem of *in vivo* adrenocortical secretion. On the other hand, it is possible (and equally likely) that the *in vitro* differences observed between steer and cow adrenals are physiologically meaningful. In which case, the data would suggest that there are either (a) differences between steers and cows with respect to their metabolism and utilization of glucocorticoids like 17-hydroxycorticosterone and corticosterone or (b) that components of the adrenocortical secretion other than 17-hydroxycorticosterone and corticosterone may be involved in the adrenocortical response to stressors. These questions merit further investigation.

Comparison of the V-L perfused cow adrenal to other in vitro studies

It is of some interest to compare the rates of corticosteroid biosynthesis, in the absence and presence of ACTH, in the perfused cow adrenal relative to other *in vitro* systems wherein ACTH effects have been reported. Table 7 summarizes the available data. Included are three studies on the rat adrenal and a single study on slices of cow adrenals; in all of these cases the adrenal tissue was incubated in Ringers solution. Comparison of our data with the study on cow adrenal slices permits an opportunity to evaluate the rate of corticosteroidogenesis achieved in the same tissue studied by two different *in vitro* methods.

a. Absolute rates of corticosteroidogenesis: In the absence of ACTH, cow adrenal tissue, whether perfused with blood or incubated as slices, produces only $\frac{1}{4}$ to $\frac{1}{5}$ as much corticosteroid per unit time as does rat adrenal tissue upon incubation. When, however, ACTH is added to the *in vitro* system in concentrations sufficient to evoke maximal stimulation, the perfused cow adrenal exhibits the highest rate of corticosteroidogenesis. The V-L

TABLE 7. COMPARISON OF THE RATE OF ADRENAL CORTICOSTEROID OUTPUT OBTAINED BY DIFFERENT *in vitro* TECHNICS IN THE PRESENCE AND ABSENCE OF ACTH

Adrenal preparation		Investigators	Corticosteroid output, μg./gm./hr.		ACTH
			Non-ACTH	ACTH	Non-ACTH
Cow	Venous perfusion	This Study	*17*	*328*	*19*
	Incubated slices	Haynes *et al.*, 1952	13	41	3.2
		Haynes *et al.*, 1954	10	43	4.3
Rat	Incubated, bisected, or quartered adrenals	Saffran and Bayliss, 1953	67	190	2.8
		Hoffman and Davison, 1953	70	~~195~~	~~2.8~~ 1.8
		Birmingham *et al.*, 1953	75	150	2.0

correction 127

perfused cow adrenal gland stimulated with ACTH produces corticosteroid at a rate 700% greater than that achieved with cow adrenal slices, and approximately 65 to 120% greater than that obtained with the incubated rat adrenal tissue.

b. Relative ACTH effect: The results described above taken together demonstrate that the ACTH effect achieved with V-L perfused cow glands represent a 19 fold increase in the rate of corticosteroidogenesis, whereas the effect of ACTH on corticosteroid biosynthesis in slices, whether of cow or rat, represents only a 2–4 fold increase. These differences in relative ACTH effect between perfusion and *in vitro* incubation of slices may be a reflection of the fact that precursors (or co-factors) present in blood are available for corticosteroid biosynthesis in perfused glands in addition to those present in adrenal tissue. In the slice experiments, the only precursor available must be present initially in the tissue or formed from the glucose present in the Ringers, solution, while other differences between these diverse *in vitro* technics may also be involved, studies carried out in our laboratories (Stone & Hechter, unpublished) demonstrate that the amount of corticosteroid formed and the relative ACTH effect is determined by the amount of blood in the system.

An objection might be raised concerning our relative ACTH effects in perfused cow glands, based upon our employment of a method which does not yield constant recovery of corticosteroid, independent of the corticosteroid level in blood perfusates. It will be recalled from Table 1 that the per cent recovery at low concentrations is approximately $\frac{1}{2}$ that obtained at high concentrations. Since the control periods of perfusion without added ACTH yield low corticosteroid output values, while perfusates following ACTH stimulation have high corticosteroid content, it is apparent that more corticosteroid, than that estimated, might have been released during the control period, in which case our relative ACTH effect might be markedly lowered. On the assumption that the recovery of the naturally occurring mixture of corticosteroids present in adrenal perfusates is similar to that obtained with cortisone (cf. Table 1) we have "corrected" our values to take this factor into account. Table 8 shows a comparison of the values, "uncorrected" and "corrected" for variable recovery of different concentrations of corticosteroid from blood by dialysis. It will be seen that this correction increases the non-ACTH values about 100%, but the ACTH values only slightly. The relative ACTH effects obtained while halved are still higher than those obtained with adrenal slices incubated in Ringers solution.

Elucidation of the reasons for differences in relative ACTH effect upon the rate of corticosteroidogenesis in diverse *in vitro* systems should contribute to further understanding of the mode of ACTH action.

TABLE 8. COMPARISON OF CORTICOSTEROID OUTPUT VALUES OF ISOLATED PERFUSED COW ADRENALS "UNCORRECTED" AND "CORRECTED" FOR VARIABLE DIALYSIS RECOVERY OF DIFFERENT CORTICOSTEROID CONCENTRATIONS

Preparation	ACTH dose	Average corticosteroid output, μg./gm./hr.		ACTH / Non-ACTH	
		Uncorrected	Corrected	Uncorrected	Corrected
V-L perfused	0	17	37	19	10
	Maximal	328	387		
Arterial perfused	0	4.6	10	10	5.4
	Maximal	47	54		

SUMMARY

The results presented demonstrate that the cow adrenal perfused with homologous citrated blood responds to added ACTH by a 10 to 19 fold increase in corticosteroid production. The magnitude of corticosteroid output, both in the absence and presence of ACTH, is markedly influenced by the method of preparing glands for perfusion. The venous cannulated lacerated gland produces about 7 times more corticosteroid per unit time per gm. gland than arterially perfused preparations in the presence of ACTH, and about 4 times more corticosteroid in the absence of ACTH. Steer glands, on the average, respond poorly to ACTH, in contrast to cow glands. The results on corticosteroid production in the perfused bovine adrenal have been compared to other *in vitro* adrenal systems.

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THE NECESSITY FOR PROGESTERONE WITH ESTROGEN FOR THE INDUCTION OF RECURRENT ESTRUS IN THE OVARIECTOMIZED EWE

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INTRODUCTION

WHILE considerable attention has been directed to the possible role of progesterone in the mating behaviour of animals, emphasis has been placed on the effects either of simultaneous estrogen-progesterone influence, or of progesterone following estrogen. The possibility that progesterone, produced by the corpus luteum of a preceding cycle, can condition an animal to respond to subsequent estrogen stimulation has received little attention. This is rather surprising, since in some species, notably the sheep and cow, ovulation without estrus is a common phenomenon (Grant, 1934; Weber, 1911; Hammond, 1946). At the onset of the breeding season in the ewe, the first ovulation is unaccompanied by estrus (Grant, 1934 and others). If ovulation is induced in the anestrous ewe, heat does not normally occur. If a second ovulation is induced 16 days later, estrus is exhibited by a high proportion of ewes which ovulate (Cole and Miller, 1953 and many others). Hammond Jr., Hammond and Parkes (1942), Hammond Jr., (1945) and Robinson (1950) have shown that the ovulation-estrus response to gonadotrophin injection in anestrum, is markedly influenced by the presence and age of a corpus luteum in the ovaries at the time of injection.

In 1945 Cole, Hart and Miller reported estrus in 39 of 48 ewes in which injection, in anestrum, with pregnant mare serum gonadotrophin (PMS) was preceded by 1 to 3 days by 40–100 mg. testosterone. More recently Robinson (1952, 1954a, 1954b) and Dutt (1952, 1953) have shown that when progesterone in injected for several days prior to PMS injection, estrus regularly accompanies induced ovulation.

This effect of progesterone might be mediated in a number of ways. For instance, it might delay the gonadotrophin induced ovulation, so prolonging the period of estrogen stimulation. Examination of ovaries has precluded this possibility (Robinson, 1954a). Hammond Jr. (1945) has suggested that uterine tone and motility may be important in estrous behaviour. In view of the 48-hour interval normally observed between estrogen

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injection and heat, Hammond suggests that proprioceptors may have to be developed in the uterus, perhaps by joint progesterone-estrogen action, before full estrus is exhibited. A third possibility is that progesterone conditions the central nervous system to respond to estrogen.

Between Oct. 1952 and Nov. 1953 an experiment, which demonstrated the necessity for both progesterone and estrogen for the maintenance of regular cycles of estrous behaviour in the ovariectomized ewe, was conducted at the State Research Farm, Werribee, Victoria, Australia. Such recurrent estrus was independent of the presence or absence of the uterus.

MATERIALS AND METHODS

Twelve Suffolk X Border Leicester-Merino yearling ewes were spayed between July 22nd and Aug. 12th, 1952. Of these, 6 were also hysterectomized.

Estrogen injections were commenced on Oct. 30th, when the behaviour of spayed ewes (Group 1) was compared with that of spayed+hysterectomized animals (Group 2). A series of 5 injections of 2 or 5 mg. estradiol benzoate at weekly intervals was followed by a 3 week spell; then followed a further series of 5 injections of 5 mg. estradiol benzoate at 14 day intervals. This interval was used subsequently as it was the most convenient approximation to the normal cycle length of 16–17 days and lay within normal physiological limits (Asdell 1946).

On Feb. 27th, 1953, the 12 ewes were regrouped into Groups A and B, each comprising 3 spayed and 3 spayed+hysterectomized animals, and the second series of injections were commenced. Each ewe received 20 injections with a 2-week period between injections. A total of 108 injections of estrogen alone and 136 injections of progesterone plus estrogen were made. Each group received a constant level of estrogen for 3 successive injections. One group was pretreated with progesterone. At the end of the 6-week period the groups were changed over; that which had been receiving estrogen only was pretreated with progesterone and *vice versa*. Estrogen levels were successively decreased at these changeovers by a factor of 0.2, from 5 mg. down to 8 μg., and then increased to 20 μg.

All injections were made intramuscularly in peanut oil. When progesterone was administered, a total of 75 mg. was given over a period of 3 days, in 6 injections each of 12.5 mg. in 1.25 ml. oil. Estradiol benzoate was given as a single injection in 1 ml. oil, and when following progesterone, was injected 40 hr. after the last injection.

Estrus was detected by running the ewes continually with 2 Suffolk rams, painted liberally on the brisket with branding fluid. The colour was changed before each estrogen injection. Ewes were examined twice daily for service, at as near as possible to 12-hourly intervals from estrogen injection. Some errors were introduced here, particularly in the winter months when the hours of day-light did not permit accurate 12-hourly observations. The time of onset of estrus, but not the duration, was determined.

RESULTS

Estrus

Weekly injections of 5 and 2 mg. estradiol benzoate resulted in a refractory condition to estrogen. After the first injection, 5 of 6 spayed ewes and 6 of 6 spayed and hysterectomized ewes came into heat. The percentage of ewes demonstrating estrus declined gradually with successive treatments

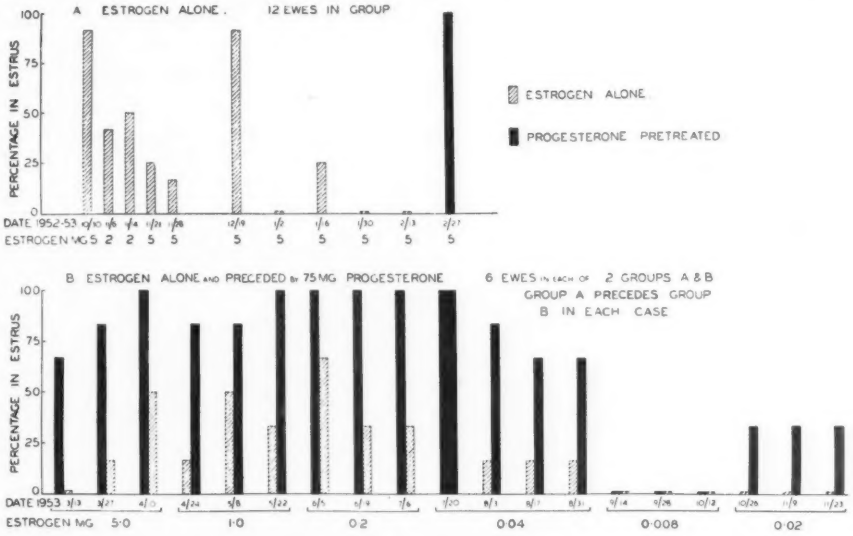
A ESTROGEN ALONE. 12 EWES IN GROUP
PERCENTAGE IN ESTRUS
ESTROGEN ALONE
PROGESTERONE PRETREATED
DATE 1952-53
ESTROGEN MG. 5 2 2 5 5 5 5 5 5 5 5
B ESTROGEN ALONE AND PRECEDED BY 75 MG PROGESTERONE
6 EWES IN EACH OF 2 GROUPS A & B
GROUP A PRECEDES GROUP B IN EACH CASE
PERCENTAGE IN ESTRUS
DATE 1953
ESTROGEN MG 5·0 1·0 0·2 0·04 0·008 0·02

FIG. 1. Estrus incidence—spayed ewes receiving repeated injections of estradiol benzoate.

and following the fifth injection only 1 spayed and 1 spayed-hysterectomized ewe showed estrus. Following an interval of 3 weeks, injection of 5 mg. produced estrus in 11 ewes. A refractory condition again developed when injections were continued at 14-day intervals, and only 4 services resulted from 48 treatments during the next 8 weeks. Presence or absence of the uterus made no demonstrable difference.

Between Feb. 23rd–25th, progesterone was injected to all ewes. On Feb. 27th, 5 mg. estrogen was injected and all ewes were served within 48 hours. The consistent induction of estrus thereafter depended entirely on whether or not progesterone preceded estrogen (Fig. 1). Of 96 cases in which progesterone preceded estrogen, between dose levels of 5 mg. and 40 μg., 86 (90%) services resulted. Comparable figures for estrogen alone were—72 injected and 21 (29%) served. At each injection, the numbers responding to progesterone-estrogen exceeded those served following estrogen alone, and no individual ewe responded regularly to estrogen unless it was preceded by progesterone.

A dose of 8 μg. estradiol benzoate was below the threshold for response for all ewes; an increase to 20 μg. resulted in 6 responses from 18 treatments in the progesterone pretreated group, and none in the estrogen only group. On the data it was not possible to determine accurate dose/response relationships. Table 1 indicates no clear dose/response relationship for estrogen alone, particularly at the higher dose levels. However, when preceded by

TABLE 1. SUMMARY OF ESTROUS RESPONSES FOLLOWING ESTROGEN AND PROGESTERONE-ESTROGEN TREATMENT

Level of estrogen mg.	Progesterone pretreated			Estrogen only		
	Total treatments	Total services	Per cent estrus	Total treatments	Total services	Per cent estrus
5	30	27	90	18	4	22
1	18	16	89	18	6	33
0.2	18	18	100	18	8	44
0.04	30	25	83	18	3	17
0.02	18	6	33	18	0	0
0.008	18	0	0	18	0	0
All Levels	132	92	70	108	21	20

progesterone there was a dose response relationship; levels of 40 μg. and above of estradiol benzoate were sufficient for maximum response; 8 μg. was below the threshold, while 20 μg. appeared marginal.

Time from estrogen to estrus

Progesterone pretreatment produced a marked advance in the time of estrus onset (Fig. 2): 50% mated within 36 hours of estrogen injection and 88% within 48 hours. When only estrogen was administered, no ewes mated within 36 hours and only 29% within 48 hours.

In neither treatment was there any suggestion of a dose/response relationship between estrogen level and time of estrus onset. Response within 36 hours was classified as "Early" and after 36 hours as "Late." The value obtained for X^2 of 15.530, d.f. = 1, indicates this advancement of estrus onset to be highly significant ($P < .001$).

The nature of estrus

When high levels of estrogen were administered alone, many ewes exhibited abnormal behaviour. They mounted other ewes, and exhibited intense sexual excitement, but would not permit service. Estrogen had a pronounced effect on behaviour, but the manifestation was abnormal. No such abnormalities were apparent when progesterone preceded estrogen; mounting behaviour was occasionally observed but such ewes were served repeatedly.

DISCUSSION

Alternate progesterone and estrogen influence is essential for the induction of recurrent estrus in the ovariectomized ewe. Furthermore, progesterone pretreatment advances the time of estrus onset following estrogen injection, regardless of the level of estrogen administered, and prevents abnormal male-like behaviour resulting from excessively high estrogen dosage. These two latter effects suggest that the role of progesterone is more

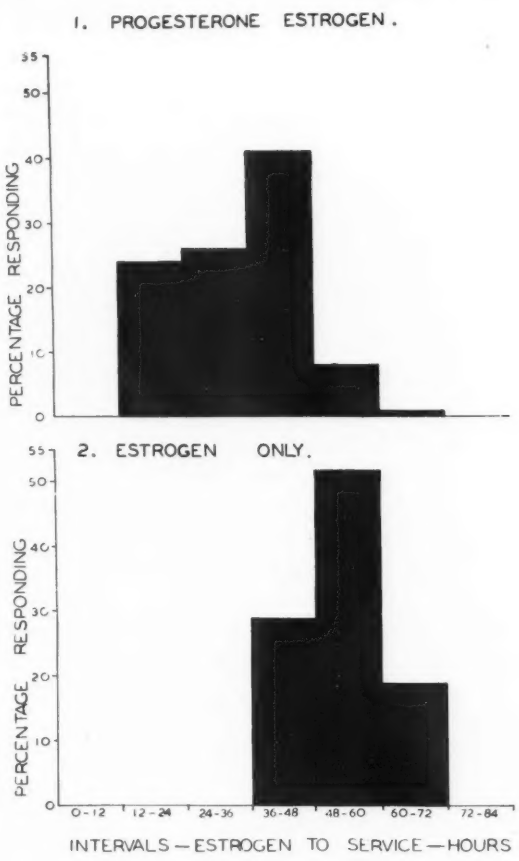

FIG. 2. Time from estrogen injection to estrus.

fundamental than merely estrogen sparing. The uterus is not involved in these phenomena.

Clearly then, normal estrous behaviour in the sheep is under dual hormonal control. Since a ewe, not conditioned by progesterone, will respond only to relatively high levels of estrogen, the phenomenon of "silent estrus" can be explained. Prior to the first ovulation of the breeding season the ewe has not been subjected to recent progesterone influence, and fails to respond to endogenous estrogen. The progesterone produced by the corpus luteum conditions the ewe to respond to the estrogen produced by the next maturing follicle. Estrus recurs with ovulation for as long as the animal is subjected to alternate progesterone-estrogen influence. If a corpus luteum fails to function adequately, or a maturing follicle fails to produce sufficient estrogen, a further "silent heat" occurs.

These several effects of progesterone need investigation in other species. No consideration of estrous behaviour in any polyestrous species can disregard the possibility that alternate progesterone-estrogen influence is necessary for full manifestation of normal female sexual behaviour.

SUMMARY

Repeated injections of high levels of estradiol benzoate (2 or 5 mg.) at 7 and 14 day intervals produced a refractory condition in 12 ovariectomized ewes (of which 6 were hysterectomized), in that few continued to exhibit normal estrous behaviour. Some animals exhibited male-like mounting behaviour. Progesterone (75 mg. in 6 twice daily injections) removed this condition and all ewes were served following estrogen injection 2 days later. The estrogen dose was reduced successively by a factor of 0.2, from 5 mg. to 8 μg. and then raised to 20 μg. Three successive injections were given at each level, at 14-day intervals. At no dose level could recurrent estrus be induced unless progesterone preceded estrogen, in which case 40 μg. approached the level for maximum response, 20 μg. was marginal, and 8 μg. ineffective. Progesterone pretreatment advanced the time of onset of estrus by 12 to 24 hours. Hysterectomy had no effect on the responses observed.

It is concluded that alternate progesterone-estrogen influence is necessary for regular cyclic estrous behaviour in the ewe.

Acknowledgments

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EFFECT OF CHRONIC PITRESSIN ADMINISTRATION ON ELECTROLYTE EXCRETION IN NORMAL DOGS AND IN DOGS WITH EXPERIMENTAL ASCITES

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MANY attempts have been made to determine the effect of posterior pituitary extract on electrolyte excretion. Conflicting reports have appeared and conclusive data on the action of pitressin on the renal tubular transport of electrolytes in the dog are lacking. The present studies were undertaken to determine the effect of chronic pitressin administration on electrolyte excretion in normal dogs and in dogs with ascites produced by constriction of the thoracic inferior vena cava. The relation of the changes in electrolyte excretion to adrenocortical function in animals with ascites has been evaluated by injection of pitressin in adrenalectomized dogs with thoracic inferior caval constriction.

METHODS

After a control period of 2–3 weeks, pitressin tannate in oil (PTO) was administered in doses of 1–2.5 units twice daily to 1) two normal dogs (dogs 1 and 2), 2) two dogs with thoracic inferior vena cava constriction, ascites and intact adrenal glands (dogs 3 and 4), and 3) two adrenalectomized dogs with thoracic inferior caval constriction and ascites (dogs 4 and 5). Dog 4 was studied before and after adrenalectomy. Observations were also made during the recovery period following pitressin administration.

The animals were kept in metabolic balance cages throughout the study except during measurements of renal hemodynamic function and blood pressure. A synthetic diet containing 80 Cal./kg./day was fed once daily. Water was allowed *ad libitum*; in addition, water was administered by stomach tube during the first course of PTO administration.

Sodium (Na), potassium (K) and water balances, plasma Na and K, hemoglobin and the postabsorptive rate of glomerular filtration (GFR) and renal plasma flow (RPF) were determined according to methods reported previously (Davis, Lindsay and Southworth, 1952). In the dogs with ascites, femoral venous and arterial pressures were also measured; the techniques are described elsewhere (Davis, Lindsay and Southworth, 1952).

RESULTS

Administration of pitressin to normal dogs resulted in a positive water balance and a gain in body weight when water was given by stomach tube

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in addition to the voluntary intake (Figs. 1 and 2). Under these circumstances, the dogs with caval constriction (dogs 3–5) showed a more positive water balance and a more rapid gain in body weight than during the control period (see Fig. 4); marked abdominal distension resulted and massive edema of the hind legs developed. A striking drop in plasma Na occurred in all dogs (Figs. 1–4); in dogs 1 and 5 plasma Na fell to 110 meq./L. Plasma K was not detectably changed except in dog 2 in which a fall from 3.5 to 2.6 meq./L. occurred. The concentration of hemoglobin in blood was consistently reduced.

After 3–5 days of PTO administration, water excretion began to increase in all but dog 5 and water balance became slightly negative; in the normal dogs body weight also fell (Figs. 1 and 2). During recovery body weight declined in the dogs with caval constriction in spite of continued Na retention (Fig. 4). In the normal dogs, after pitressin was discontinued body weight fell further to a level below the control weight. The concentration of plasma Na and K (dog 2) and the hemoglobin content of the blood returned to the control level during recovery.

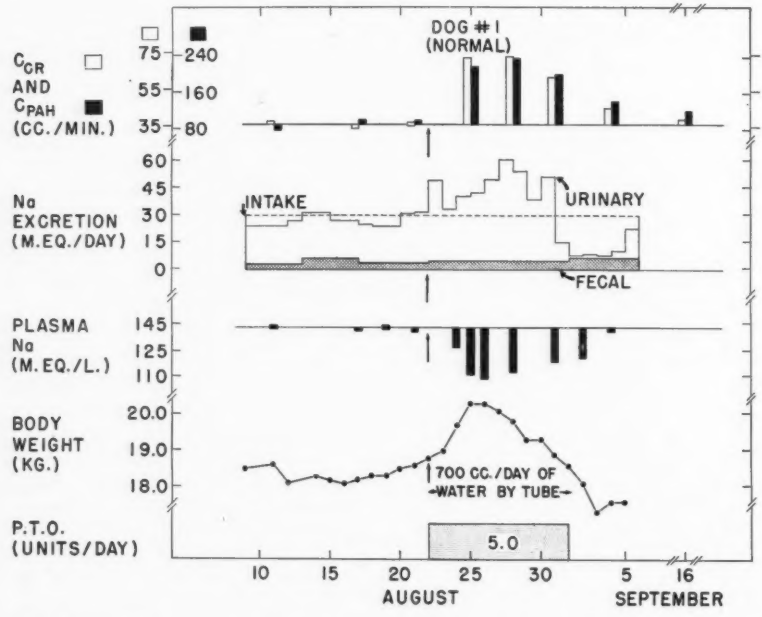

FIG. 1. Effect of administration of pitressin tannate in oil (PTO) on renal hemodynamic function, Na excretion, plasma Na and body weight in a normal dog. Values for C_{CR}, C_{PAH} and plasma Na are plotted as columns above and below horizontal lines which represent control levels.

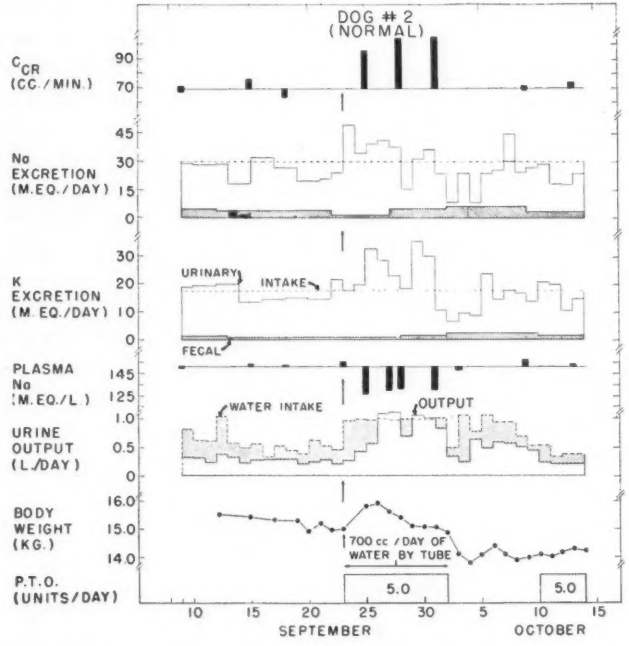

FIG. 2. Glomerular filtration rate (C_{CR}), Na and K excretion, plasma Na, urine output and body weight during pitressin (PTO) administration to a normal dog. During the first course of PTO, water was administered by stomach tube whereas water intake was limited to drinking during the second period of PTO injection. C_{CR} and plasma Na are plotted as solid columns from average control values.

After the first course of PTO, dogs 1, 2 and 5 were given the same dose of pitressin but water intake was voluntary (Figs. 2 and 4). A slight reduction in water ingestion occurred and water balance was achieved at a lower level of water exchange; body weight, plasma Na and the hemoglobin content of blood were not detectably altered.

In the presence of forced water administration, a marked increase in renal hemodynamic function occurred in all animals (Figs. 1–4) except dog 4 following adrenalectomy. When water was not administered by stomach tube, GFR and RPF remained at the control levels (Figs. 2 and 4).

The response in electrolyte excretion to pitressin during administration of water by gastric tube varied in the three different types of animals. In the normal dogs (Figs. 1 and 2), Na and K balances were negative and the increase in electrolyte excretion was entirely urinary. In the dogs with thoracic inferior vena caval constriction, ascites and intact adrenal glands, both urinary and fecal Na excretion which was low during the control period showed a further significant drop in dog 3 (Fig. 3) but no detectable change

occurred in dog 4. In both dogs 3 (Fig. 3) and 4, total K balance became slightly negative as a result of a marked elevation in fecal K output; urinary K excretion fell. It should be pointed out that the decline in Na excretion and the greater increase in fecal K output occurred in dog 3 in which Na retention was less complete and fecal K excretion was lower during the control period than in dog 4. During maintenance of the adrenalectomized dogs with caval constriction and ascites on 1 mg./day of desoxycorticosterone acetate (DCA), pitressin failed to alter the rate of Na and K excretion (Fig. 4). In dog 5 (Fig. 4), the dose of DCA was reduced to 0.5 mg./day but adrenal insufficiency developed before adequate data were obtained. During the recovery period, Na and K balances were positive for several days in the normal animals. In dogs 3 and 4, recovery was accompanied by a fall in fecal K excretion. When water ingestion was limited to voluntary intake, no alterations in electrolyte excretion occurred (Figs. 2 and 4).

In the dogs with thoracic caval constriction, femoral arterial pressure remained at the control level during pitressin administration except during development of adrenal insufficiency in dog 5 after the dose of DCA had

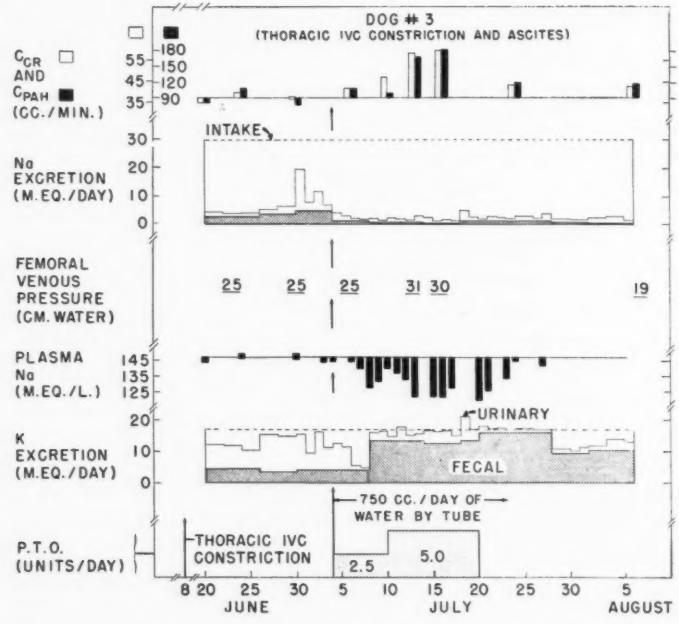

FIG. 3. Alterations in renal hemodynamic function, Na and K excretion, femoral venous pressure and plasma Na produced by pitressin (PTO) in a dog with thoracic inferior vena cava (IVC) constriction and ascites. See Fig. 1 for the convention used in plots of C_{CR}, C_{PAH} and plasma Na.

been reduced to 0.5 mg./day. Femoral venous pressure increased in dogs 3–5 (see Fig. 3).

DISCUSSION

The present observations with pitressin show that in the dog significant water retention occurred only during forced water administration. During voluntary ingestion of water, the small reduction in water intake may have been a reflection of slight water retention at the onset of PTO administration and the consequent development of decreased thirst; thereafter water intake remained low and fluid retention failed to occur. Failure of water retention to occur on a low fluid intake has also been reported in man (Leaf and associates, 1953).

After 3–5 days of PTO administration, water excretion increased in all

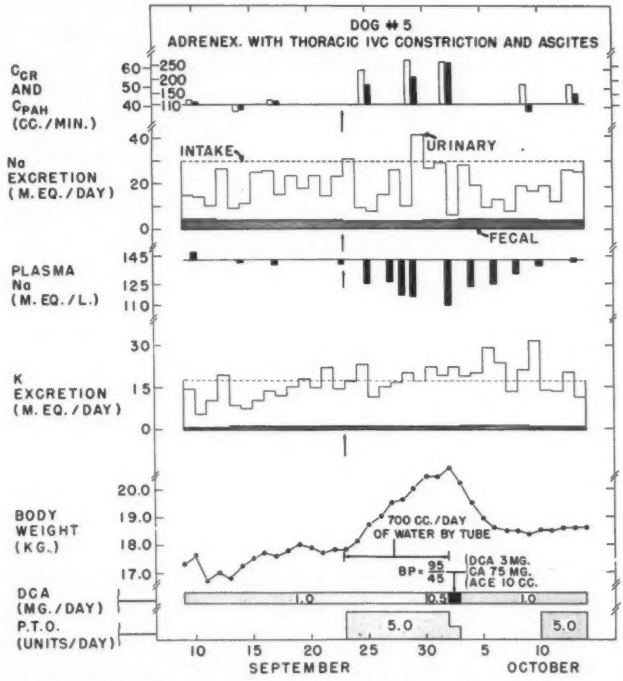

FIG. 4. Effect of pitressin (PTO) in an adrenalectomized (adrenex) dog with thoracic inferior caval constriction and ascites during maintenance on desoxycorticosterone acetate (DCA). PTO was given with (first course) and without (second course) administration of water by gastric tube in addition to drinking. On the last day of the first course of PTO, evidence of adrenal insufficiency appeared and additional therapy (75 mg. of cortisone acetate (CA) and 10 cc. of aqueous adrenal cortex extract (ACE)) were given. See Fig. 1 for the convention used in plots of C_{CR}, C_{PAH} and plasma Na.

but dog 5 and the balance became slightly negative. Available data do not provide an explanation for the "escape" from water retention. An osmotic diuresis seems unlikely; net K loss was too low to suggest sufficient excretion of nitrogen to effect an appreciable osmotic action. Cessation of water retention during chronic pitressin administration has not been reported in man (Leaf, et al. 1953; Harris and coworkers, 1953; Weston and associates, 1953) or rat (Silvette, 1941).

In spite of the increase in water excretion, a marked expansion of the extracellular fluid and plasma volume was sustained for several days. This increase in body fluid is shown by the fall in plasma Na, drop in hemoglobin, gain in body weight and positive water balance. In the normal dogs, increased Na excretion contributed to the hyponatremia but in the dogs with ascites the low plasma Na cannot be related to external Na loss since Na balance was positive. Water entered body cells and osmotic equilibrium was established at a lower level of effective osmotic pressure.

The striking increase in renal hemodynamic function may be related to the expanded extracellular fluid and plasma volume. It is generally held (Smith, 1951) that the volume of extracellular fluid and plasma is an important factor in regulation of the renal circulation. Failure of GFR and RPF to increase during pitressin injection in the absence of forced water administration demonstrates that pitressin had no direct influence on renal circulatory function. Association of increased renal clearances of endogeneous creatinine and inulin with water retention during chronic pitressin administration has been described in man (Leaf, *et al.*, 1953).

The increase in urinary Na and K excretion in the normal dogs was accompanied by a marked elevation in GFR which may explain the natriuresis and kaluresis. The response in Na excretion was similar to that reported for normal subjects (Leaf, *et al.*, 1953; Harris, *et al.*, 1953; Weston, *et al.*, 1953) but increased K excretion was not described in these studies in man. In the present observations, the negative K balance was of sufficient magnitude to indicate movement of K from cells to the extracellular compartment. The data obtained during voluntary water intake show that pitressin had no direct effect on renal tubular transport of electrolytes; Na and K balances were unchanged.

In dogs with ascites and intact adrenal glands, the changes in Na and K excretion which accompanied pitressin administration were similar to those reported previously (Davis, Howell and Southworth, 1953) to be suggestive of an increase in circulating adrenocortical salt retaining hormones. A decline in urinary and fecal Na excretion occurred in dog 3 but no detectable change occurred in dog 4 possibly because of the lower rate of excretion during the control period in this animal. However, in dogs 3 and 4 fe-

cal K output increased markedly while urinary K excretion showed a definite fall. The marked degree of Na retention and pattern of K excretion which were present during pitressin administration were observed in a previous study (Davis, Howell and Southworth, 1953) of adrenalectomized dogs with thoracic inferior vena caval constriction with large doses only (25 mg./day) of DCA. It appears, therefore, that adrenocortical salt retaining activity was augmented in the dogs with ascites and intact adrenal glands. This increased activity occurred through some functional alteration secondary to water retention. Failure of electrolyte excretion to change in adrenalectomized dogs with thoracic caval constriction (dogs 4 and 5) during maintenance on a constant amount of DCA may indicate that functional adrenocortical tissue is essential for the response to pitressin in dogs with experimental ascites.

SUMMARY AND CONCLUSIONS

The effects of chronic pitressin administration (1-2.5 units of PTO twice daily) have been studied in 1) two normal dogs, 2) two dogs with ascites produced by thoracic inferior vena cava constriction, and 3) two adrenalectomized dogs with thoracic inferior caval constriction and ascites. Water retention was apparently the primary action; this effect and the subsequent changes occurred only when water was administered by stomach tube in addition to voluntary intake. After 3–5 days of fluid retention, water excretion increased and no further expansion of the volume of plasma and extracellular fluid occurred. A striking hyponatremia resulted in all animals. A marked increase in GFR and RPF occurred in all but one dog (an adrenalectomized animal). In the two normal dogs, a natriuresis and kaluresis resulted but in the dogs with ascites and intact adrenal cortices Na excretion remained low or decreased further while fecal K output increased. In the adrenalectomized dogs with ascites, Na and K excretion was unaltered during maintenance on 1 mg./day of DCA. It is suggested that the changes in Na and K excretion in the dogs with ascites and intact adrenal glands are indicative of an increase in adrenocortical salt retaining activity. When water intake was limited to drinking in the two normal animals, no changes in electrolyte excretion were detected; this indicates that pitressin had no direct effect on renal tubular transport of electrolytes.

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METABOLIC COMPETITION BETWEEN DESOXYCORTICOSTERONE ACETATE AND STROPHANTHIN-G IN THE RAT[1]

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SINCE there is a degree of structural similarity between the cardiac aglucones and the sodium-retaining steroids of the adrenal cortex, there are theoretical grounds for the existence of a metabolic competition. Experimental data bearing on such a possible relationship are desirable for elucidation of the mechanism of action of the cardiac glycosides. The observations which will be described relate to interactions between desoxycorticosterone acetate (DCA) and ouabain (strophanthin-G) in the intact rat, with special reference to organ weights, tissue electrolyte concentrations and adrenal histology.

METHODS

Male, albino rats of the Sprague-Dawley strain, with a weight range of 175–230 g., were placed on an *ad libitum* complete purified diet which contained 30 mM. Na and 6 mM. K per 100 g. dry diet. The composition of this diet has been given in a previous publication (Gardner *et al.*, 1952). The animals were maintained in a constant temperature room at 78° F. Ouabain was selected for use because of its water-solubility.[3] The effects of both a "therapeutic" and a "toxic" dose of ouabain were assessed as follows: electrocardiograph tracings with a Sanborn Viso-Cardiette were made on rats receiving graded amounts of ouabain injected subcutaneously in aqueous solution. The largest dose of ouabain which still did not cause electrocardiographic changes was 1.7 mg. per kg. This was taken as the therapeutic dose level and is designated as DIGa. When the dosage was increased to 3 mg. per kg. striking electrocardiographic changes were seen, as shown in Figure 1. This was taken as the toxic dose level and is designated as DIGb. On a logarithmic scale the latter dose is twice the former dose. Rats receiving the larger dose of ouabain exhibited transient paralysis of their hind legs after each injection, a

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[3] Ouabain was furnished by S. B. Penick and Co., New York, N. Y.

TABLE 1. DESIGN OF EXPERIMENT SHOWING GROUP DESIGNATIONS AND TREATMENTS FOR CONTROL AND EXPERIMENTAL RATS. THERE ARE SIX RATS IN EACH GROUP. ALL TREATMENTS WERE GIVEN SUBCUTANEOUSLY

Group No.	Designation	Treatment
I	Control	None
II	DCA	Desoxycorticosterone acetate, 5 mg. once per day
III	DIGa	Ouabain, 1.7 mg./kg./twice per day
IV	DIGb	Ouabain, 3.0 mg./kg./twice per day
V	DCA+DIGa	As above
VI	DCA+DIGb	As above

phenomenon described by others (Gold *et al.*, 1947). The relative resistance of rats to cardiac glycoside therapy has long been noted (Lendle, 1935).

Thirty-six rats were divided into 6 groups as shown in Table 1. The control animals received no injections. The rest received ouabain in aqueous solution, DCA[4] in aqueous suspension or both by subcutaneous injection as outlined in Table 1. Treatment was continued for 30 days, at the end of which time the rats were sacrificed by decapitation. Organ weights were obtained and serum, skeletal muscle, and cardiac muscle were prepared for chemical analysis and analyzed according to techniques described in previous publications (Gardner *et al.*, 1950; Gardner *et al.* 1952). Adrenal glands were fixed in 10% formalin, after which they were studied in frozen sections stained for lipids with sudan black B.

RESULTS

(a) *Organ and Body Weights:* Table 2 shows the data obtained on organ weights, expressed as milligrams per 100 gm. final body weight. The renal weight changes were most striking. Kidneys from the rats receiving DCA with or without ouabain weighed more than the controls by significant amounts. Mean adrenal weights in rats receiving ouabain with or without DCA were elevated. There were no significant differences ($P \leqq 0.02$) among any of the groups in the weights of testes and thymus. The hearts from the DCA+DIGa group weighed significantly more than the control hearts. Only the animals receiving the smaller dose of ouabain (III) had final body weights not significantly less than those of the control group.

(b) *Analytic Data on Serum:* Table 3 shows the data which were obtained from the chemical analyses of serum samples. None of the groups showed significant differences in values for serum total calcium or magnesium. The groups receiving DCA+ouabain showed a slight but significant lowering of the serum inorganic P. All the experimental groups showed serum Cl values significantly lower than the control group. However, by far the lowest serum Cl values were found in the DCA-treated group (II), the next lowest in the two DCA+ouabain groups and the nearest normal values in the two groups receiving ouabain alone.

[4] Desoxycorticosterone acetate was supplied by Ciba Pharmaceutical Products, Summit, N. J.

TABLE 2. MEAN VALUES FOR ORGAN WEIGHTS ON CONTROL AND EXPERIMENTAL RATS. THE DATA ARE EXPRESSED AS MILLIGRAMS PER 100 GRAMS FINAL BODY WEIGHT

No.	Group	Heart	Kidneys (2)	Adrenals (2)	Testes (2)	Thymus
I	Control	321	768	15	1057	168
	S.E.	8	20	0.2	72	25
II	DCA	353	1093*	15	1197	165
	S.E.	7	86	1	40	16
III	DIGa	298	772	18.7	1096	138
	S.E.	21	13	1	27	18
IV	DIGb	311	726	20*	1221	137
	S.E.	28	12	1	48	5
V	DCA+DIGa	375*	1144*	19*	1186	141
	S.E.	16	43	0.8	59	7
VI	DCA+DIGb	330	1018*	19	1182	164
	S.E.	32	63	2	54	18

S.E. = Standard error of the mean = $\sqrt{\frac{\Sigma\Delta^2}{N(N-1)}}$.

Figures marked with an asterisk (*) differ significantly ($P \leqq 0.02$) from values of corresponding control group.

(c) *Analytic Data on Skeletal Muscle:* Table 4 shows chemical data obtained from analyses done on skeletal muscle. Skeletal muscle Na concentration was highest in the three groups receiving DCA, whether DCA was

TABLE 3. MEAN VALUES FOR DETERMINATIONS ON SERUM OF CONTROL AND EXPERIMENTAL RATS

No.	Group	Cl	P	Ca	Mg
		mEq./l.	mg./100 ml.	mg./100 ml.	mEq./l.
I	Control	99	8.6	7.8	1.8
	S.E.	0.7	0.1	0.7	0.04
II	DCA	72*	7.9	8.3	1.8
	S.E.	0.6	0.4	0.2	0.1
III	DIGa	94*	8.9	9.1	1.8
	S.E.	1	0.2	0.3	0.1
IV	DIGb	94*	8.1	7.9	1.8
	S.E.	1	0.2	0.9	0.03
V	DCA+DIGa	80*	7.5*	8.7	1.8
	S.E.	1	0.2	0.6	0.07
VI	DCA+DIGb	84*	7.5*	8.2	1.9
	S.E.	3	0.2	0.1	0.06

P = Inorganic phosphorus.
S.E. = Standard error of the mean.
Figures marked with an asterisk (*) differ significantly ($P \leqq 0.02$) from values of corresponding control group.

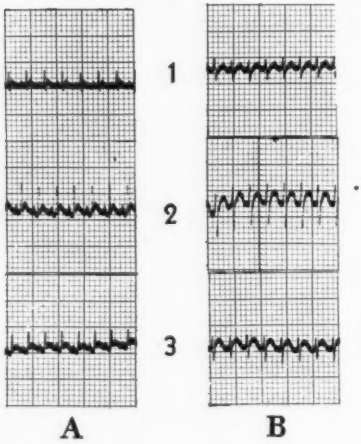


FIG. 1. Electrocardiographic tracings done on a rat before (A) and 40 minutes after (B) the animal received 3.0 mg./kg. of ouabain subcutaneously. The numbers 1, 2, and 3 refer to the three standard electrocardiograph leads.

given alone or in combination with ouabain. Potassium concentration was lowest in the group treated with DCA alone; not so markedly depressed in the groups receiving DCA+ouabain, and slightly but significantly lowered in the two groups receiving ouabain alone. Skeletal muscle Mg values were significantly lowered in the group receiving the larger dose of ouabain.

TABLE 4. MEAN VALUES FOR DETERMINATIONS ON SKELETAL MUSCLE OF CONTROL AND EXPERIMENTAL RATS. THE DATA ARE EXPRESSED PER KILO OF FAT-FREE, WET TISSUE

No.	Group	Water	Na	K	Ca	Mg	Cl
		gm.	mEq.	mEq.	mEq.	mEq.	mEq.
I	Control	758	35	96	4.5	23.0	10.9
	S.E.	2	1	1	0.2	1.4	0.9
II	DCA	726	54*	66*	10.7*	20.5	12.4
	S.E.	25	1	1	0.8	0.8	0.4
III	DIGa	757	45*	87*	8.7*	22.3	7.4*
	S.E.	2	1	0.9	0.8	0.3	0.6
IV	DIGb	734	34	82*	10.1*	17.6*	7.6*
	S.E.	7	1	0.6	1.0	0.8	0.5
V	DCA+DIGa	761	50*	74*	7.0*	19.3	7.6*
	S.E.	5	2	1	0.5	0.2	0.5
VI	DCA+DIGb	745	54*	72*	11.2*	19.5	9.0
	S.E.	18	1	3	0.7	1.3	0.6

S.E. = standard error of the mean.
Figures marked with an asterisk (*) differ significantly ($P = \leq 0.02$) from values of corresponding control group.

TABLE 5. MEAN VALUES FOR DETERMINATIONS ON CARDIAC MUSCLE OF CONTROL AND EXPERIMENTAL RATS. THE DATA ARE EXPRESSED PER KILO OF FAT-FREE WET TISSUE

No.	Group	Water	Na	K	Ca	Mg
		gm.	mEq.	mEq.	mEq.	mEq.
I	Control	778	40	89	7.0	22.1
	S.E.	8	1	0.8	0.4	0.8
II	DCA	779	47*	77*	5.4	19.6
	S.E.	4	1	0.9	1.1	0.9
III	DIGa	799	38	88	8.3	15.8*
	S.E.	21	2	1	1.5	1.3
IV	DIGb	773	43	84	7.1	16.5*
	S.E.	5	1	2	1.1	0.2
V	DCA+DIGa	794	49	85	8.8	14.7*
	S.E.	9	3	1	2.1	0.8
VI	DCA+DIGb	807	39	85	7.9	16.3*
	S.E.	21	4	1	0.4	0.8

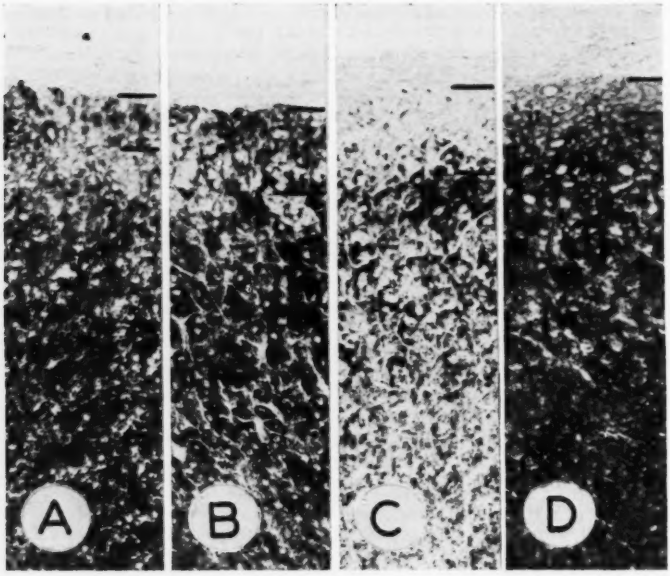
S.E. = standard error of mean.
Each determination was made on heart muscle pooled from two rats. Accordingly N = 3 in the calculation of S.E. for each group in this table only.
Figures marked with an asterisk (*) differ significantly ($P \leq 0.02$) from values of corresponding control group.

Muscle chloride values were decreased in all four groups receiving ouabain. The water content of skeletal muscle was similar in all groups.

(d) *Analytic Data on Cardiac Muscle:* Table 5 shows the chemical data on this tissue. Sodium concentration was significantly elevated in the DCA group (II) and K values were significantly lowered in this group. The group receiving the smaller dose of ouabain (DIGa) had myocardial K concentrations which did not differ from the controls. The group getting the greater dose of ouabain (DIGb) showed myocardial K concentrations somewhat lower than the controls and with more scatter. This latter range of K concentration was also found in the two ouabain+DCA groups, i.e. they were not as depressed as the DCA group, but still not quite as high as the control group. Myocardial Mg values were lowered in all four groups which received ouabain but not in the group treated with DCA alone. Values for myocardial Ca and water concentration did not vary in any of the groups.

(e) *Histology of Adrenal Cortices:* Figure 2 demonstrates the histological findings.[5] DCA alone induced both atrophy and depletion of the glomerulosa (cf. Greep and Deane, 1947). Ouabain alone produced enlargement of this zone as well as an increase in its lipid content. When DCA and ouabain were given together the atrophy seen with DCA alone was not observed, although the lipid content of the glomerulosa cells was lower than normal in most of the animals.

[5] A preliminary report of these findings has been published (Deane and Gardner, 1951).



EXPLANATION OF FIG. 2

Frozen sections of the adrenal cortex, stained with sudan black B for lipids. All ×200. On the right of each photomicrograph the width of the zona glomerulosa is indicated by two lines.

(A) Control rat (I). The glomerulosa measures 40 μ in width and contains a moderate amount of lipid.

(B) Rat receiving the larger dose of ouabain only (IV). The glomerulosa is enlarged (60 μ) and contains a moderate amount of lipid.

(C) Rat receiving the larger dose of ouabain plus desoxycorticosterone acetate (VI). The glomerulosa is broad (65 μ) though depleted of lipid droplets.

(D) Rat receiving desoxycorticosterone acetate only (II). The glomerulosa measures 30 μ and is entirely free of lipid droplets.

DISCUSSION

(a) *Animals Receiving Only DCA:* There was a fall in myocardial K and increase in myocardial Na. The observed effects of DCA on body and organ weight, adrenal weight and histology as well as on the chemical findings in serum and skeletal muscle are in agreement with previous observations (Durlacher *et al.*, 1942; Greep and Deane, 1947; Darrow *et al.*, 1948).

(b) *Animals Receiving Only Ouabain:* There was an increase of mean values for adrenal weight (mg. per 100 gm. body wt.) in all groups receiving ouabain (Table 2). Histologically there was enlargement of the adrenal glomerulosa under ouabain treatment. Both ouabain groups (III and IV) showed lowered concentrations of skeletal muscle K. The fall of K concen-

tration in heart muscle was less impressive than in skeletal muscle. This finding of moderate K depletion in animals treated chronically with a cardiac glycoside is similar to observations made by several other workers with a variety of physiological preparations (Calhoun and Harrison, 1931; Cattell and Goodell, 1937; Wood and Moe, 1938, 1940, 1942; Hagen, 1939; Wedd, 1939). These data thus contribute toward clarifying the findings that the low potassium state potentiates the toxic effects of digitalis (Sampson *et al.*, 1943; Lown *et al.*, 1951; Levine *et al.*, 1952). Although it has been reported that cardiac glycosides have actions similar to adrenal cortical hormones (Zwemer *et al.*, 1940; Hadju and Szent-György, 1952), the observations are not supported by the present findings or other recent work (Chen *et al.*, 1952).

Magnesium concentration was significantly lowered in heart muscle at both dose levels of ouabain, and in skeletal muscle at the higher dose level. Recalling the physiological antagonism between Ca and Mg, the foregoing findings may throw light on the observations that the injection of Ca salts has a toxic synergism with cardiac glycoside therapy (Smith *et al.*, 1939) and the finding that injected Mg salts abolish certain electrocardiographic manifestations of cardiac glycoside toxicity (Szekely and Wynne, 1951).

(c) *Animals Receiving Ouabain+DCA:* The rats which received both DCA and ouabain, as in the groups receiving only ouabain, had adrenal weights greater than the controls. There was also failure of the adrenal glomerulosa to undergo the histological atrophy found with DCA therapy (II). Skeletal and cardiac muscle K concentrations in these two groups were depressed, but not so low as in the rats treated with DCA alone. The ouabain+DCA groups had cardiac muscle K concentrations no lower than the rats who receved only ouabain. In skeletal muscle there appeared to be a less effective inhibition by ouabain of the K-depleting effect of DCA than there was in cardiac muscle (see Table 4). These findings indicate that in the presence of ouabain there was partial to complete inhibition of the K-depleting effect of DCA on skeletal and cardiac muscle. This inhibitory mechanism appears to operate independently of the moderately K-depleting effect of ouabain given alone.

Heart muscle Mg, as in the animals receiving ouabain alone, was decreased in concentration in the ouabain+DCA groups. Thus, while ouabain appeared to interfere with several aspects of DCA effect, DCA did not seem to influence the Mg-depleting action of ouabain on cardiac muscle.

The above findings suggest that there is a metabolic competition between ouabain and DCA, somehow related to the adrenal cortex. Whether such an interaction takes place by molecular coupling of ouabain to DCA, reminiscent of the digitonin-cholesterol combination, or through spatial dis-

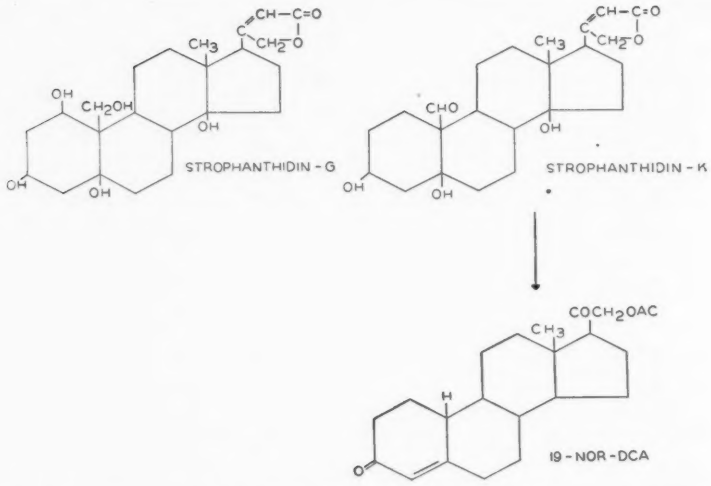


FIG. 3. In the upper left is shown the structural formula of strophanthidin-G, the aglucone of strophanthin-G (ouabain). On the right is depicted the transformation of strophanthidin-K into 19-nor-desoxycorticosterone acetate (Ehrenstein, 1944).

placement of DCA by ouabain, is not disclosed by the present study. It is of interest that Ehrenstein (1944) found strophanthidin-K a convenient starting point for the synthesis of 19-nor-desoxycorticosterone acetate (Fig. 3). The latter compound is a close analog of DCA, differing only in the substitution of hydrogen for the methyl group at C-19. Enzymatic transformation of digitalis aglucones in the animal body could result in the production of such structural analogs, thus providing the setting for metabolic competition.

SUMMARY

To explore the possibility of interaction between desoxycorticosterone acetate (DCA) and ouabain in the intact animal, adult rats were given daily injections of DCA, ouabain, or both together for a period of 30 days:

1) DCA therapy alone resulted in a marked fall of K concentration and rise of Na concentration in skeletal and cardiac muscle, together with atrophy of the adrenal glomerulosa and depletion of lipid droplets in that zone.

2) Ouabain therapy resulted in enlargement of the adrenal glomerulosa, with an increase in its lipid content. There was a marked fall of Mg concentration and moderate fall of K concentration in skeletal and cardiac muscle.

3) DCA+ouabain therapy resulted in changes generally similar to those found when only ouabain was given. This group showed partial to com-

plete inhibition of the various DCA effects. The results suggest that a metabolic antagonism exists between DCA and ouabain in the rat.

Acknowledgment

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STUDIES OF ACTH ACTION UPON PERFUSED BOVINE ADRENALS: MINIMAL ACTH CONCENTRATION REQUISITE FOR MAXIMAL GLANDULAR RESPONSE[1]

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INTRODUCTION

IN THE previous paper, the response of the venous cannulated-lacerated cow adrenal (V-L gland), perfused with citrated homologous blood to maximal concentrations of ACTH was described (Macchi and Hechter, 1954). In the present work, various concentrations of ACTH were perfused through isolated V-L cow adrenal glands. By comparing the corticosteroid output response of different glands perfused with a constant concentration of ACTH, it has been possible to obtain an estimate of the minimal ACTH concentration required to produce maximal rate of corticosteroid biosynthesis in this system.

METHODS

Cow adrenal glands (weighing between 15 and 27 gm.) were utilized in these studies. Venous cannulated-lacerated glands were perfused at a blood flow rate of 1 liter per hour, independent of gland weight, with homologous citrated blood at temperatures of $38° \pm 1°$ C. The adrenal effluent was removed from the system, and was not permitted to recirculate through the gland. The perfusion apparatus employed was a dual system which included a multicycle circuit for the preliminary preparation of rat liver "filtered" blood, and a single cycle circuit designed to permit the passage of individual small volumes of perfusion medium through an adrenal gland under relatively constant hydrostatic pressure. The apparatus employed for the latter purpose is diagramatically illustrated in Figure 1. Briefly described, this latter system consisted of a glass reservoir bulb appropriately fitted to permit oxygenation, mixing, and introduction of freshly prepared medium which was transferred to the reservoir as needed. The circuit ensures that the medium was filtered through a double Fenwal stocking type nylon filter prior to per-

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fusion of the cannulated gland. The general design of the experiments was as follows: the adrenal glands were first perfused with blood for 30 minutes unless otherwise stated in order to warm and stabilize the preparation and then was followed with an additional 300 ml. of blood. The second perfusate was analyzed for corticosteroid in order to obtain the corticosteroid output in the absence of added ACTH. Thereafter, the gland was perfused continuously for varying intervals with a single concentration of ACTH, during which period individual samples at 18 minute intervals were collected. This experimental design permitted analysis of corticosteroid output during different intervals of the experiment.

Glands were studied at concentrations of 0.001, 0.01, 0.1 and 10 I.U. ACTH per liter

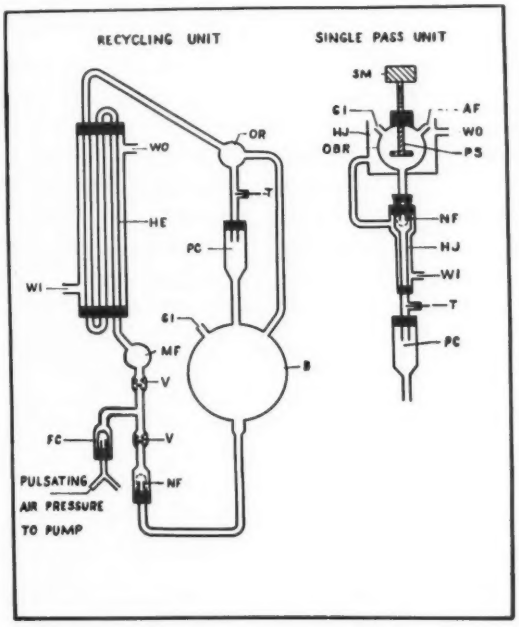

FIG. 1. Schematic drawing of the perfusion apparatus used to study the influence of ACTH concentration upon the corticosteroid output of the isolated cow adrenal. Each portion of the apparatus labelled according to key letters supplied in the diagram is defined as follows: WI, WO—inlets and outlets to permit circulation of water at constant temperature through heating jackets (HJ) and around heating elements (HE) of stainless steel tubing; FC—rubber finger cot to provide pulsatile pressure; MF—Miller-type plexiglass filters; V—one-way glass valves; NF—Fenwal nylon stocking-type filter; OR—organ chamber; GI—inlet for admitting hydrated gas; BR—blood reservoir to contain and to permit oxygenation of blood; OBR—reservoir to contain blood for single cycle perfusion; SM—stirrer motor and PS—paddle-type stirrer for blood mixing; AF—opening for addition of fresh perfusion medium and GI—inlet for admitting hydrated gas (both openings are fitted with rubber tubing so that they extend above the level of the water in the heating jacket); T—thermometer.

blood. A single ACTH preparation was used (Lot #K55808) which was kindly supplied by the Armour Co. Since ACTH is partially inactivated upon incubation in blood (Pincus *et al.*, 1952), ACTH was added at 18 minute intervals to samples of warmed blood in an attempt to maintain the concentration at or near the desired level throughout the period of perfusion.

The methods of determining the corticosteroid content of perfusates and of evaluating corticosteroid output rates have been described previously (Macchi and Hechter 1954). The corticosteroid output rates to be presented are not corrected for variable recovery of steroid from perfusates by dialysis, since we were interested in relative and not absolute differences obtainable at different concentrations of ACTH.

RESULTS

Level of corticosteroid output during varying intervals of perfusion with ACTH

In order to study the influence of time upon the response of glands perfused with ACTH, V-L cow adrenals were perfused with a single concentration of ACTH for 72 minutes, and the corticosteroid output in excess of the control (non-ACTH) value was measured at consecutive 18 minute intervals. Table 1 illustrates the results obtained with ACTH concentrations of 0.001, 0.01, 0.1 and 10 I.U. per liter blood. It will be seen that with all ACTH concentrations studied, the maximal response of the adrenal to stimulation with corticotrophin is not achieved in the first 18 minute period. Maximal production of corticosteroid occurs in the perfusion intervals

TABLE 1. CORTICOSTEROID OUTPUT OF THE V-L COW ADRENAL PREPARATION PERFUSED WITH SINGLE DIFFERENT CONCENTRATIONS OF ACTH

ACTH conc., I.U./liter	Gland No.	Net corticosteroid output at various perfusion intervals, μg./gram adrenal/hour			
		0–18′	18–36′	36–54′	54–72′
0.001	1	−95	−44	7	−10
	2	93	103	89	98
	3	−18	66	44	58
	Av.	− 5	42	47	49
0.01	4	118	95	170	95
	5	142	186	216	214
	6	169	238	279	76
	7	34	89	123	38
	Av.	116	152	197	104
0.1	8	189	364	418	377
	9	172	313	233	163
	10	67	158	219	239
	Av.	143	278	290	260
10	11	153	211	271	226
	12	238	200	314	302
	13	162	211	212	189
	Av.	184	207	266	239

of 18–36 or 36–54 minutes after initiation of ACTH treatment. After achieving maximal response, there appears to be a tendency for the corticosteroid output to decline during the last period of perfusion (54–72 min.) which is seen most clearly with the 0.01 I.U. per liter dosage of ACTH. The data shown in Table 1 suggest that the magnitude of corticosteroid output response may be related to the concentration of ACTH used for perfusion. Statistical evaluation of the differences observed with the various ACTH concentrations studied did not appear to be justifiable in view of the limited data on hand. Instead, an additional number of glands were perfused with certain ACTH concentrations to enable us to evaluate the data statistically.

ACTH Dosage-Response relationship

The response to various ACTH concentrations was measured during a single perfusion interval in these experiments. The perfusion interval of 18–36 minutes after initiation of treatment with ACTH was selected because (a) it was the earliest period when all ACTH concentrations studied had produced a positive *mean* response; (b) the response during this period was not significantly different from the maximal response of glands during any subsequent 18 minute interval and (c) corticosteroid output expressed as μg./gm. gland per hour during this single 18 minute period is of the same approximate order as that achieved during the over-all period of perfusion for 72 minute with all concentrations of ACTH studied.

Table 2 shows the net corticosteroid output values (in excess of the control non-ACTH value) obtained with varying concentrations of ACTH during the perfusion interval 18–36 minutes after stimulation. It will be seen that in the dosage range from 0.001 to 0.1 I.U. ACTH per liter, the magnitude of response is proportional to the log concentration of ACTH. A further 100 fold increase in ACTH concentration does not increase the response. Our data indicate, therefore, that 0.1 I.U. ACTH per liter is the minimal dose which elicits a maximal response. Statistical evaluation by means of Fisher's t test of the differences in response obtained with the various ACTH doses studied are shown in Table 3. It will be seen that each of the responses obtained in the range from 0.001 to 0.1 I.U./liter is statistically different from that obtained with the preceding dose.

Effect of ACTH upon glands, previously perfused with blood for several hours

The results described above were obtained with glands arbitrarily perfused with blood for 48 minutes before testing the effect of ACTH. The question arose whether the latent period observed before the perfused adrenal responds maximally to a particular dose of ACTH might be an expression of incomplete "resynthesis" of depleted metabolic components

TABLE 2. RELATIONSHIP BETWEEN ACTH CONCENTRATION AND CORTICOSTEROID OUTPUT OF THE V-L COW ADRENAL PREPARATION

ACTH conc., I.U./liter	Gland No.	Net corticosteroid output 18–36′ interval, μg./gram adrenal/hour
0.001	1	−44
	2	103
	3	66
	4	−87
	5	24
	6	99
	7	−12
	Av.	21
0.01	8	95
	9	186
	10	238
	11	124
	12	89
	Av.	146
0.1	13	364
	14	313
	15	158
	Av.	278
10	16	211
	17	200
	18	211
	19	143
	20	225
	21	292
	22	165
	23	307
	24	362
	25	278
	Av.	239

which are assumed to occur as the result of perfusion with blood in the pre-ACTH period of the experiment.

To obtain evidence on this point, 9 glands were perfused with blood for 156 minutes (instead of the usual 48 minute interval) and the response to 10 I.U. per liter ACTH was measured. Table 4 shows the per cent increase

TABLE 3. STATISTICAL EVALUATION OF DIFFERENCES IN CORTICOSTEROID OUTPUT OBTAINED WITH VARIOUS ACTH CONCENTRATIONS DURING THE 18–36 MINUTE PERFUSION INTERVAL

ACTH concentrations compared, I.U./liter	Fisher's t	P
0.001 vs. 0.01	3.13	<0.02–>0.01
0.01 vs. 0.1	2.59	<0.05–>0.02
0.01 vs. 10	2.54	<0.05–>0.02
0.1 vs. 10	0.84	>0.40

TABLE 4. EFFECT OF EXTENDED BLOOD PRETREATMENT UPON THE LATENT PERIOD REQUIRED TO ACHIEVE MAXIMAL CORTICOSTEROID OUTPUT WITH MAXIMAL EFFECTIVE DOSES OF ACTH†

Blood pretreatment (minutes)	Gland No.	Per cent increase in corticosteroid output during 18–36 minute perfusion interval
48	1	93
	2	82
	3	136
	4	38
	5	0
	6	30
	Av.	63*
156	7	0
	8	0
	9	83
	10	137
	11	0
	12	68
	13	9
	14	7
	15	11
	Av.	35*

* $P > 0.10$.
† 10 I.U. ACTH per liter.

in corticosteroid output obtained during the 18–36 minute interval following ACTH treatment as compared to that obtained during the 0–18 minute interval with glands pretreated by perfusion with blood for 48 and 156 minutes respectively. It will be observed that the latent period of response is not abolished by prolonged pretreatment with blood. The mean percentage increase in net corticosteroid output obtained during the 18–36 minute interval over the initial 18 minute period of perfusion is of the same order of magnitude in both groups; the differences noted are not statistically significant. As an incidental finding it was observed that the mean corticosteroid output of 9 glands perfused with blood for 156 minutes prior to ACTH was 351 μg./gm./hr. as compared to the value of 343 μg./gm./hr. in the glands pretreated with blood for 48 minutes. These data demonstrate unequivocally that the responsiveness to ACTH of the isolated cow adrenal perfused with blood is fully maintained at least for a period of two hours. The tendency toward a decline in corticosteroid output observed with certain ACTH concentrations (cf. Table 1) is therefore not explicable in terms of exhaustion of the glandular capacity to produce corticosteroids as a consequence of perfusion alone.

DISCUSSION

Our *in vitro* data suggest that the minimal concentration of ACTH in the perfusion medium required to produce maximal adrenal response in the V.L perfused cow gland lies between 0.01 and 0.1 I.U. ACTH per liter. It is

possible to calculate from the *in vivo* data in man, that the concentration of ACTH required to stimulate effectively the human adrenal is of this range of magnitude. The average human may be assumed to weigh 70 kgm. and to have a blood volume of about 5 liters which is fully recirculated every 5 minutes. Renold *et al.* (1952) have shown in humans that the continuous intravenous infusion of 20 I.U. of ACTH over an 8 hour period produces maximal adrenal response as indicated by 17-ketosteroid excretion. On the basis of our assumptions of the "average" human, the effective concentration of circulating ACTH would be of the order of 0.04 I.U. per liter. Forsham *et al.* (1951) have shown that intravenous administration of 1 μg. (0.025 I.U.) per minute of a polypeptide ACTH produces maximal stimulation of the human adrenal cortex *in vivo* as evaluated by estimation of metabolic changes (*e.g.*, eosinopenia, urinary steroid excretion, changes in carbohydrate and electrolyte metabolism). If one assumes that 5 minutes is required for mixing, the concentration of ACTH is (0.025 I.U.×5/5 liters) 0.025 I.U. per liter. The *in vitro* data on perfused isolated glands where the minimal effective concentration lies between 0.01 and 0.1 I.U. per liter is thus in excellent agreement with the *in vivo* data in man concerning the ACTH concentration required for maximal stimulation.

While in excellent agreement with the *in vivo* studies cited, our data, in marked contrast, are in disagreement with other *in vitro* studies. The concentrations of ACTH necessary to effect maximal (or near maximal) corticosteroid biosynthesis in the bisected rat adrenal (Saffran and Bayliss, 1953) and in cow adrenal slices (Haynes *et al.*, 1954) incubated in Ringers' solution are shown in Table 5. It will be seen that adrenal slices, whether from cow or rat, require about 400 times more ACTH (per unit volume of medium) than does the perfused cow adrenal to achieve maximal (or near maximal) corticosteroid production. A similar conclusion results if the comparison is made on the basis of I.U. required per unit weight (ignoring the volume of the medium). Since the magnitude of corticosteroid response in ACTH stimulated adrenal tissue is greater in the perfused gland than

TABLE 5. COMPARATIVE DATA ON ACTH CONCENTRATION REQUIRED TO ATTAIN MAXIMAL OR NEAR MAXIMAL CORTICOSTEROID OUTPUT IN VARIOUS *in vitro* SYSTEMS

Type of *in vitro* preparation	Investigator	ACTH, I.U./liter
V-L perfused bovine adrenal	This study	0.10
Incubated bovine adrenal slice	Haynes *et al.* (1954)	>64*
Incubated rat adrenal	Saffran and Bayliss (1953) Birmingham *et al.* (1953)	38† 50–100

* Maximal output not achieved with this concentration, presumably larger amounts would be necessary to produce maximal stimulation.

† Estimated value calculated from the data of Saffran and Bayliss.

in the incubated slices (Macchi and Hechter, 1954), the high sensitivity of the perfused gland to ACTH is not achieved at the expense of a poor corticosteroidogenic response.

The different ACTH requirements of the isolated perfused cow adrenal relative to cow slices indicates that the effectiveness of ACTH in these two *in vitro* systems is entirely dissimilar. In the latter case, the ACTH added in large part may be ineffective because ACTH (a) may be destroyed in part by protelytic enzymes liberated from the sliced tissue or (b) does not effectively penetrate the slice to act upon all of the adrenocortical cells. Further work is necessary to clarify this discrepancy between these two types of *in vitro* preparations.

While it is generally accepted that *in vivo* the adrenal responds rapidly to ACTH (Bush, 1953) and indeed within seconds in the isolated perfused gland (Hechter, 1951), the results of this investigation demonstrate that there is a latent period of at least 18 minutes before the maximal effect of ACTH is achieved in the isolated perfused cow adrenal.

SUMMARY

Using the venous cannulated-lacerated cow adrenal preparation, it has been shown that 0.001 I.U. ACTH per liter evokes a definite increase in corticosteroid production. Increasing concentrations of ACTH increase the magnitude of response and maximal corticosteroid production is achieved at 0.1 I.U. ACTH per liter. The ACTH effect at all concentrations has a latent period of about 18 minutes before maximal corticosteroid response is obtained. The relation of these findings to other *in vitro* systems and to appropriate *in vivo* data are discussed.

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STUDIES OF ACTH ACTION UPON PERFUSED BOVINE ADRENALS: DURATION OF ACTH ACTION[1]

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INTRODUCTION

IN THE previous study (Macchi and Hechter, 1954b) on the venous cannulated-lacerated (V-L) cow gland, it was shown that the magnitude of corticosteroid production is a function of the ACTH concentration of the perfusion medium. The question arises whether the effect of ACTH upon corticosteroid biosynthesis depends primarily upon maintenance of an effective concentration of circulating ACTH or whether other factors are involved. Thus, the possibility exists that circulating ACTH is fixed and retained in the adrenal (as has been suggested by Sonenberg, *et al.*, 1951) so that glands might continue to exhibit increased corticosteroidogenesis despite the withdrawal of ACTH from the circulation. To obtain information on this point we have studied the rate of corticosteroid production in cow adrenal glands after withdrawal of ACTH from the perfusion medium.

METHODS AND RESULTS

Cow glands perfused via the adrenal vein were used for these experiments. The methods employed for perfusion and for evaluation of corticosteroid production both in the absence and presence of ACTH have been described fully in the previous papers of this series (Macchi and Hechter, 1954a, 1954b). The ACTH utilized in these studies was an Armour preparation (ACTHar-A-Blend 2H). As in our previous report, all glands were perfused at a flow rate of 1 liter per hour without recirculation of the medium. They were first perfused for 48 minutes with blood (not containing added ACTH) to stabilize the preparation and to obtain the control level of corticosteroid output in the absence of added ACTH.

(a) *Persistence of ACTH effect:* To determine whether glands continue to produce corticosteroid at increased rates following withdrawal of ACTH, cow glands were perfused for 18 minutes with blood containing maximal concentrations of ACTH. A dose of

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[1] This investigation was aided in part by the Research and Development Board, Office of the Surgeon General, Department of the Army, under Contracts DA-49-007-MD-255 and DA-49-007-MD-260. Portion of thesis submitted by I. A. Macchi to the graduate school of Boston University, Department of Biology, in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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1.0 I.U. per liter was selected since this dose is 10 times greater than the minimal concentration necessary to evoke maximal corticosteroid response (Macchi and Hechter, 1954b). Following treatment with ACTH, the gland was then perfused with 2.4 liters of blood, not containing added ACTH over a period of 144 minutes. The post-ACTH perfusates of separate intervals corresponding to 18–36, 54–72, 90–108 and 126–144 minutes were collected and analysed separately. The corticosteroid output values were compared to those obtained during the initial stimulation period with ACTH. The perfusate collected during the first period of perfusion following ACTH (0–18 minutes) consisted of 300 ml. of blood which was collected separately but was not analyzed. This initial perfusion was utilized to "wash out" any residual exogenous ACTH from the perfusion apparatus.

Table 1 shows the results of three experiments. It will be seen that in all cases perfusion with ACTH increased the corticosteroid production, and that following withdrawal of ACTH, the level of corticosteroid output declined. Although the absolute levels varied in these different glands, the mean percentage decline from the ACTH treatment period is consistent. It will be seen from Table 1 that approximately 45% of the corticosteroid output attained during ACTH stimulation is still evident 18–36 minutes after withdrawal of exogenous ACTH. This declines in exponential form, so that after 2 hours the glandular corticosteroid output while still somewhat in excess of the control (non-ACTH) value is only 18% of the rate attained during the period of stimulation with ACTH. These data demonstrate the persistence of an ACTH action after removal of ACTH from the circulating medium. It should be emphasized, however, that corticosteroid production at or near maximal levels requires maintenance of an adequate concentration of circulating ACTH.

(b) *Effect of prolonged perfusion with minimal effective doses of ACTH:* If circulating ACTH is fixed in adrenal tissue, prolonged perfusion with minimal effective concentrations might lead in time to accumulation of sufficient amounts of ACTH to activate corticosteroid production in the adrenal. In our previous work, (Macchi and Hechter, 1954b) it was shown that perfusion with 0.001 I.U. ACTH per liter leads to a slight increase in corticosteroid output which is not statistically significant. This dose of ACTH was therefore administered continuously for a period of 126 minutes to determine whether a significant effect upon corticosteroid output might be achieved by prolonged perfusion. Table 2 shows the results obtained upon perfusing 7 glands with 0.001 I.U. ACTH per liter for a period of 126 minutes. Corticosteroid output was evaluated at suc-

TABLE 1. CORTICOSTEROID OUTPUT OF THE V-L PERFUSED COW ADRENAL AT VARIOUS TIME INTERVALS AFTER REMOVAL OF ACTH FROM THE CIRCULATION

ACTH conc. I.U./liter	Perfusion time interval after ACTH withdrawal (minutes)	Experiment No. 1		Experiment No. 2		Experiment No. 3		Average	
		C.O.	% Diff.	C.O.	% Diff.	C.O.	% Diff.	C.O.	% Diff.
1	0	407	100	582	100	324	100	438	100
0	18–36	200	49	237	41	149	46	195	45
	54–72	147	36	127	22	89	28	121	28
	90–108	90	22	104	18	68	21	87	20
	126–144	71	17	89	15	70	22	77	18

C.O.—Refers to net corticosteroid output expressed as μg./gram adrenal/hour.
% Diff.—Refers to per cent of the initial corticosteroid output obtained during ACTH stimulation.

cessive 18 minute intervals. ACTH was added to the blood used for perfusion at 18 minute intervals to minimize the possible inactivation of ACTH by bovine blood (Pincus *et al.*, 1952). It will be seen that prolonged perfusion with this dose of ACTH does not increase corticosteroid production significantly. Any possible effect of this dosage occurs 18–36 minutes after stimulation and does not increase with increasing perfusion time. If ACTH is accumulated by the perfused adrenal, our data demonstrate that the amount retained is insufficient to activate corticosteroid biosynthesis in a significant manner. It is important to note that after 126 minutes of perfusion with blood, the isolated adrenal exhibits a normal corticosteroidogenic response to ACTH stimulation (Macchi and Hechter, 1954b). This demonstrates that the failure to achieve high levels of corticosteroid production in this study cannot be ascribed to glandular exhaustion consequent to prolonged periods of perfusion.

DISCUSSION

The results of these studies demonstrate that the isolated perfused cow adrenal stimulated with maximal concentrations of ACTH exhibits a partial retention of the ACTH effect after removal of circulating ACTH from the system. This residual glandular activity upon perfusion with blood declines in an exponential fashion. Corticosteroid production, relative to that attained with maximal ACTH stimulation, was 45% at 18–36 minutes and 18% at 126–144 minutes after withdrawal of ACTH from the system. Similar findings have been reported by Saffran and Bayliss (1953) in incubated rat adrenal tissue. Evaluation of their data indicates a similar magnitude of decline with time following withdrawal of ACTH. The persistence of ACTH activity, demonstrated with two independent *in vitro* technics, might result if (a) ACTH in the medium were retained and fixed by the adrenal tissue (*via* adsorption) or (b) ACTH "triggers" a reaction involved in biosynthesis which once initiated then proceeds for a time in the absence of ACTH. The available evidence strongly suggests that the mode of *in vitro* action of ACTH in corticosteroid biosynthesis is to regulate the rate of cholesterol transformation to progesterone; the subsequent steps in-

TABLE 2. CORTICOSTEROID OUTPUT OF THE V-L PERFUSED COW ADRENAL DURING PROLONGED PERFUSION WITH A MINIMAL EFFECTIVE DOSE OF ACTH

ACTH conc. I.U./liter	Gland No.	Net corticosteroid output of various perfusion intervals, μg./gram adrenal/hour				
		0–18′	18′–36′	36′–54′	54′–72′	126′–144′
0.001	1	−95	−44	7	−10	10
	2	93	103	89	98	72
	3	18	66	44	58	69
	4	− 6				4
	5	2				0
	6	− 4				17
	7	4				10
	Av.	− 4	42	47	49	26

volved in corticoid production have been shown to be dependent upon enzymatic systems which are not under the direct influence of ACTH (Hechter, 1953; Stone and Hechter, 1953; Stone *et al.*, 1953). If progesterone (or an earlier intermediary) accumulated in the adrenal during the period of ACTH stimulation, it is possible that continued production at an accelerated rate might occur despite the absence of ACTH in the system. The duration of such an effect would then depend upon the degree of postulated accumulation of the early intermediate in the corticosteroidogenic sequence.

Alternatively, one may consider "fixation" of ACTH by the adrenal. The data of Sonenberg *et al.* (1951) with ACTH preparations labeled with I^{131} demonstrate that the adrenal gland accumulates labelled material. As recognized by Sonenberg *et al.*, their findings are not definitive with regard to ACTH since the labelled ACTH preparation administered was not homogeneous. The possibility remains that the accumulated radioactivity represented inactive contaminants instead of labelled ACTH. Our attempt to demonstrate accumulation by prolonged perfusion with small concentrations of ACTH was negative. This, however, does not preclude the possibility of accumulation. Perhaps had other doses or conditions been tested it might have been possible to demonstrate such a phenomenon.

It is thus clear that the available evidence does not permit differentiation between these basically dissimilar mechanisms which were suggested to account for a persistence of ACTH activity obtained under *in vitro* conditions. It is important to emphasize, however, that the duration of such effects are extremely limited in time. Within two hours, most of the corticosteroidogenic effect of previous ACTH treatment is gone. For the steady production of corticosteroid at high rates in the isolated perfused gland a constant supply of circulating ACTH is requisite. This aspect of ACTH action upon the adrenal provides a regulatory mechanism for corticosteroid production which is responsive to fluctuations in the level of circulating ACTH. The physiological consequences of a situation where the gland retains the effect of ACTH stimulation for a prolonged period of time independent of circulating ACTH would be to have a system which is basically insensitive to regulation to meet the varied requirements of the organism for corticosteroids. The decline in corticosteroid production observed following withdrawal of ACTH must be related to the possible mechanisms of persistence discussed above. Either the ACTH is removed by washing and/or by enzymatic inactivation or alternatively the intermediate which is postulated to be accumulated as the result of ACTH action becomes exhausted.

The clinical data on ACTH in man do not permit precise comparisons

with our *in vitro* perfusion data. However, one of the basic findings in human adrenal activation studies which has emerged from clinical studies is that stimulation of the adrenal is best achieved by *continuous infusion* of small concentrations of ACTH, rather than by injection of large single doses of ACTH (Gordon *et al.*, 1951; Renold *et al.*, 1951; Mandel *et al.*, 1951; McIntosh *et al.*, 1951). The necessity for a constant supply of circulating ACTH for maximal adrenal stimulation in the human is thus in basic agreement with the similar findings in this *in vitro* study.

SUMMARY

Following stimulation of the venous cannulated-lacerated cow adrenal preparation with maximal concentrations of ACTH, there is a retention of a portion of the ACTH effect despite its removal from the circulation. The residual activity upon corticosteroid output declines in an exponential manner. Corticosteroid production (relative to that attained with ACTH) was 45% at 18–36 minutes and 18% at 126–144 minutes after withdrawal of ACTH from the system. The significance of these findings is discussed briefly.

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HYPERGLYCEMIC AND GLYCOGENOLYTIC ACTIVITY OF PREPARATIONS FROM HUMAN URINE[1]

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THE presence of a hyperglycemic agent in extracts of urine from human subjects has been repeatedly reported (Houssay and Biasotti, 1933; Dingemanse and Kober, 1933; Mather, Katzman and Doisy, 1933–34; Harrow, Naiman, Chamelin and Mazur, 1934). Houssay *et al.* (1933) were able to demonstrate the presence of this activity more frequently in the urine of diabetic patients than in specimens from normal men. On the other hand Werch and Altschuler (1937) were not able to detect hyperglycemic activity in urine from normal individuals. Some of the early workers (Dingemanse *et al.*, 1933; Mather *et al.*, 1933), found this activity associated with gonadotrophic material, but purified gonadotrophins were later shown to be free of hyperglycemic activity (Davis, Hinsey and Markee, 1934). These latter authors suggested that the hyperglycemic effect of urinary preparations could be due to salts of uric or hippuric acids, which they demonstrated to be hyperglycemic when injected into rabbits. More recently Meduna and Vaichulis (1948) have reported that the urine of certain schizophrenics contains greater amounts of a hyperglycemic agent than the urine of normal men and women. This observation has been confirmed, and the presence of two different hyperglycemic factors in urine has been reported (Walker and Mayer-Gross, 1951). The two hyperglycemic agents are urates and a substance apparently associated with the amino acid content of the specimen.

The possibility that the hyperglycemic activity of urinary preparations could be due to the presence of pancreatic glucagon (Bürger, 1937; Fòa, 1953; Sutherland and Cori, 1948), prompted the writers to investigate the properties of this urinary factor. While this work was in progress, Saka (1952) presented evidence showing that ". . . the substance isolated from the diabetic urine may be of pancreatic origin." The experiments reported below are incompatible with this tentative conclusion, and the evidence to

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be presented shows that the urinary agent is unlike any of a number of known glycogenolytic factors previously investigated, including glucagon.

METHODS AND RESULTS

Male New Zealand white rabbits weighing between 2 and 4 kg. were used, all animals being fed the same commercial diet (Purina). The substances injected were dissolved in 4 ml. of isotonic phosphate buffered saline (pH 7.4), prepared as indicated below. *In vitro* incubations were carried out in open test tubes (150 mm. length×22 mm. inside diameter), air being the gas phase. The incubation medium was prepared by mixing 5 parts of isotonic saline with one part of 0.11 M potassium phosphate buffer (pH 7.4). Tissue slices were cut with the aid of a Stadie-Riggs microtome, and tissue suspensions were prepared in conventional glass homogenizers. Glucose was determined by the method of Nelson (1944) using Folin-Wu sugar tubes. Tissue glycogen was separated and hydrolyzed by the method of Good, Kramer, and Somogyi (1933). The glucose content of the glycogen hydrolysate was determined by the method of Nelson using as standards of reference glucose solutions containing the same acid concentration as the diluted glycogen hydrolysates. All glycogen values will be expressed as glucose equivalents. Uric acid was determined by the method of Brown (1945).

Preparation and properties of urinary concentrate. Crude concentrates of the urine of normal men and women were prepared by dialysis and lyophilization. Untreated 24-hour specimens were dialyzed against cold running tap water for 18–24 hours, and dialysis continued for 24 hours against cold distilled water with repeated changes. The impermeate was concentrated by blowing air around the suspended dialyzing bags at room temperature and the resulting concentrate was lyophilized. The weight of solid material obtained varied from 300–700 mg. in seventeen concentrates prepared by this procedure, and the nitrogen content was usually about 7%.

The intravenous administration of this residue to rabbits, at a dosage of 20 or 40 mg./kg. of body weight, caused the blood glucose concentration to increase by about 70 mg./100 ml. as may be seen from the representative experiments in Figure 1. This response was elicited by an amount of concentrate equivalent to approximately 1/20th of the average daily output of solid residue. Since the intravenous administration of gelatin (40 mg./kg. body weight) had no effect on the level of blood glucose, and the administration of ACTH (corresponding to 10 mg. Armour Standard LA-1-A per kg. body weight) had only a slight effect, it is concluded that the hyperglycemic action of the urinary concentrate is not a non-specific effect. All extracts tested were found to be hyperglycemic.

The hyperglycemic activity is at least partially destroyed by heating an aqueous solution of an active preparation at pH 5.5–6 in a boiling water bath for one hour (Fig. 1).

The presence of urinary concentrate stimulated the release of glucoese into the medium by rabbit liver slices incubated aerobically. Figure 2 shows that this activity is roughly proportional to the dose employed. The results illustrated in this figure show that with this particular preparation a maximal glucose output was obtained with 500 to 600 μg, per ml. of

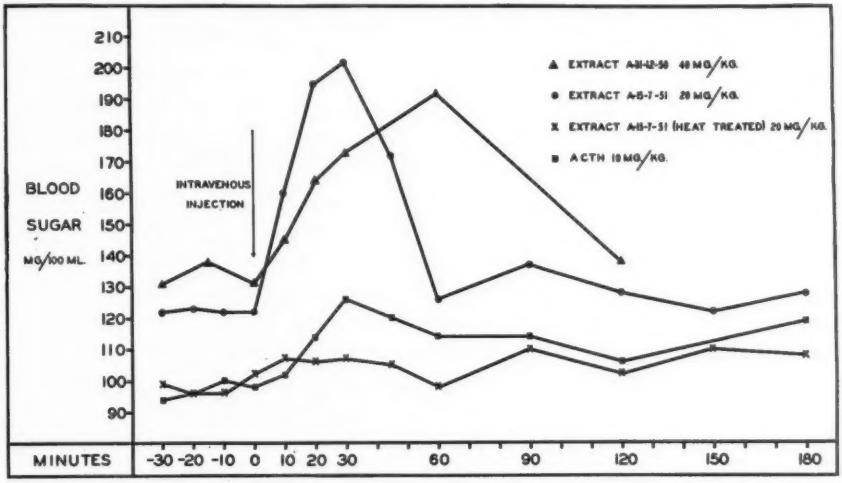

FIG. 1. Blood glucose changes following intravenous injection of urinary concentrates and ACTH in normal rabbits. In each case the material injected was dissolved in 4 ml. of phosphate-buffered isotonic saline (pH 7.4).

incubation medium and a measurable effect with as little as 50 μg./ml.

The active material could be precipitated from aqueous solutions of the crude concentrate by saturating with ammonium sulphate. The activity of the crude concentrates was not completely destroyed following 3 hours incubation at 37° C. in 0.08 N sodium hydroxide.

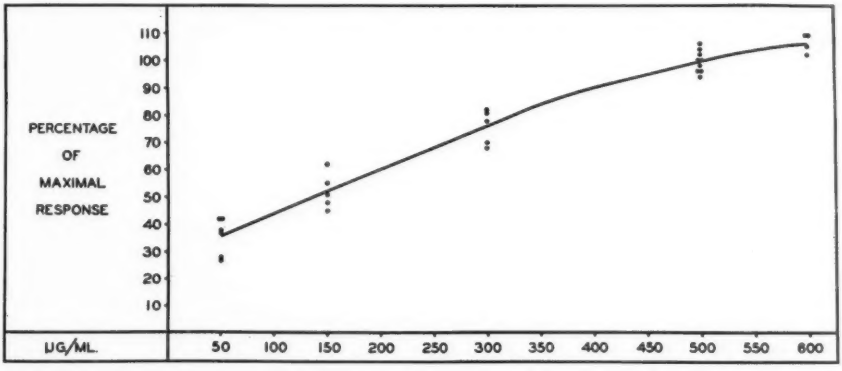

FIG. 2. Changes in glucose content of the medium of rabbit liver slices incubated with various amounts of urinary concentrate dissolved in 1.2 ml. of phosphate-buffered isotonic saline. Incubation was performed at 37° C. for 60 minutes and shaking at a rate of 120 oscillations per minute. The results are expressed as percentage of maximal response.

Purification of urinary hyperglycemic agent. The addition of ethanol to acidified urine precipitated a very active glycogenolytic and hyperglycemic fraction. The experiments reported below were carried out on alcohol-insoluble material prepared by the following procedure. After filtering the specimen, the pH of the urine was adjusted to 5 by the addition of 10% hydrochloric acid; two volumes of 95% ethanol were added and the mixture stood at 4° C. for 24 hours. The precipitate was collected by centrifugation, suspended in 100 ml. distilled water and stirred mechanically for 30 minutes. The insoluble material was removed by filtration and discarded, the filtrate was dialyzed for 24 hours against running cold water and 24 hours against distilled water with repeated changes. The lyophilized impermeates weighed from 50 to 150 mg. 24-hour specimen and the nitrogen content was about 5%. The preparations were negative to the Hopkins-Cole test, either in solution or in dry form, and the xanthroproteic test was also negative. A strongly positive reaction was observed in the Millon-Nasse test and a faint blue coloration with biuret reagent.

The unpurified alcohol-insoluble material was found to contain uric acid; however, following overnight dialysis it was no longer possible to detect the presence of uric acid even in relatively concentrated solutions of the purified factor (100 mg./100 ml.).

The purified *alcohol precipitate* from *urine* (APU) was found to be approximately 8 times as active, on a weight basis, as the crude urinary concentrate in inducing the release of glucose by rabbit liver slices. In doses of 0.5 or 1.0 mg./kg. body weight, APU increased blood glucose in the rabbit by 30 to 40 mg. %.

Action of APU on glycogen content of liver slices. In an effort to standardize the assay procedure, it was decided to investigate the tissue glycogen changes during the incubation of liver slices, in view of the possibility that glycogen changes might be more consistent than the changes in glucose secretion. It was found that the APU-induced increase in glucose output (i.e. the glucose output of APU-treated slices minus the output of untreated control slices) was not always accompanied by a corresponding additional decrease in liver glycogen; this is shown by Experiments 1 to 3 in Table 1. The total glycogen disappearance, however, exceeded the total glucose output. In some instances (Experiments 8 to 11, Table 1) the net glycogen disappearance due to APU was considerably smaller than the additional increase in glucose secretion by the slice. Only in four experiments out of 11 (Experiments 4 to 7), did sufficient additional glycogen disappear to account for the rise in glucose output. The positive differences in Table 1 represent apparent increases in carbohydrate and the additional glucose presumably could be derived either from gluconeogenic processes or from diminished utilization of carbohydrate by the tissue. The ability of the

TABLE 1. ACTION OF URINARY GLYCOGENOLYTIC FACTOR ON GLYCOGEN CONCENTRATION AND GLUCOSE OUTPUT BY LIVER SLICES[1]

Experiment	1	2	3	4	5	6	7	8	9	10	11
Glycogen Decrease[2]	−0.6	−0.7	−0.8	−14.0	−7.8	−4.3	−3.1	−3.1	−1.8	−1.9	−9.4
Glucose Increase[3]	3.3	6.2	3.8	4.0	2.9	2.5	3.2	4.4	4.4	4.2	12.4
Difference	+2.7	+5.5	+3.0	−10.0	−4.9	−1.8	+0.1	+1.3	+2.6	+2.3	+3.0

[1] Values represent the average of duplicate determinations.
[2] Mg. glycogen (as glucose)/gm. tissue. Net decrease after subtracting decrease of untreated slices.
[3] Mg. glucose in medium/gm. tissue. Net increase after subtracting increase of untreated slices.

tissue to increase its glucose output, while preserving its glycogen stores either wholly or in part, could not be correlated with initial glycogen content since this behavior was observed in tissues in which the glycogen concentration at the beginning of incubation ranged between 3 and 9%.

Action of APU or glucagon on liver homogenates. The possibility was then considered that the pancreatic and urinary hyperglycemic factors were two different substances, perhaps acting through different mechanisms. Sutherland and Cori (1948) reported that glucagon did not increase the glucose output of liver homogenates, and the present writers confirmed this finding using crude pancreatic extracts. It was decided therefore to determine what effect, if any, APU had on glycogenolysis and glucose output by liver homogenate. Slices and homogenates were prepared from the same liver and incubated either in the presence of 10 μg./ml. of glucagon[3] or APU (100 μg./ml.); untreated tissue preparations were simultaneously incubated for one hour. The results of three different experiments appear in Table 2, from which it can be seen that, whereas glucagon is essentially inactive in stimulating glucose release by liver homogenates, APU greatly increases glycogenolysis by this tissue preparation.

Under the effect of APU, liver homogenates lost 81 to 98% of the initial glycogen content, as opposed to decreases ranging between 40 and 53% in slices treated in the same manner. Another difference in the manner of action of glucagon and APU is shown by the changes in glycogen content and glucose output by slices. In all three experiments the net decrease in glycogen content under the effect of glucagon exceeded the net increase in glucose output, while the inverse was true in the last two experiments when slices were treated with APU. A fourth experiment presented in Figure 3 illustrates this difference in behavior. Both treatments produced approxi-

[3] Dr. Otto K. Behrens of Eli Lilly & Company kindly supplied the glucagon preparation, 208-41B-277.

TABLE 2. CHANGES IN TISSUE GLYCOGEN AND GLUCOSE OUTPUT BY LIVER SLICES AND HOMOGENATES TREATED WITH PANCREATIC OR URINARY HYPERGLYCEMIC FACTORS

Tissue Preparation	Slice[1]		Homogenate[2]	
Treatment	Glucagon	APU	Glucagon	APU
Dose μg./ml.	10	100	10	100
Glycogen Change[3]	−10.1	−4.9	−4.0	−61.8
Glucose Output[4]	+ 8.1	+4.5	+0.1	+19.2
Difference	− 2.0	−0.4	−3.9	−42.6
Glycogen Change	− 7.1	−3.3	−0.6	−64.0
Glucose Output	+ 6.0	+6.4	−0.3	+20.5
Difference	− 1.1	+3.1	−0.9	−43.5
Glycogen Change	− 4.9	−3.4	+2.5	−59.9
Glucose Output	+ 2.4	+4.6	+0.8	+19.7
Difference	− 2.5	+1.2	+3.3	−40.2

[1] Each figure represents the average of four determinations.
[2] Each figure represents the average of two determinations.
[3] Mg. glycogen (as glucose)/gm. tissue. Net change after subtracting decrease of untreated tissue.
[4] Mg. glucose in medium/gm. tissue. Net increase after subtracting increase of untreated tissue.

mately the same increase in glucose release, but the additional glycogen disappearance induced by APU could only account for half of this increase in medium glucose.

Attempts to identify APU. The possibility has been considered that the active glycogenolysis induced by APU in liver homogenates could be due to one of a number of naturally-occurring substances that are known to induce or aid glycogenolysis or hyperglycemia. None of the following substances induced glycogen disappearance when added to liver homogenates: uric acid (20 μg./ml.), growth hormone (100 μg./ml.),[4] pitressin (100 μg./ml.).[4] Adenosine-5-phosphoric acid in a concentration of 100 μg./ml. induced a slight glycogen disappearance corresponding to 1–3 μg. of APU.

Chaput and Heard (1951) have reported that in the presence of calcium (0.6 mM final concentration which is equal to 24 μg. Ca/ml.) liver slices secrete increased amounts of glucose into the medium. Table 3 presents the results of experiments in which the effect of both calcium and APU was determined on the glycogen and glucose changes in slices and homogenates prepared from the same liver.

It can be seen that at comparable dose levels calcium is less effective than APU in stimulating the release of glucose by liver slices and that the glycogenolytic action of calcium ion on homogenates is negligible.

Preliminary test for enzymatic activity of APU per se. To investigate the

[4] Dr. D. A. McGinty of Parke Davis & Company kindly supplied the preparations of growth hormone, RXO99862 and pitressin, Lot 119615.

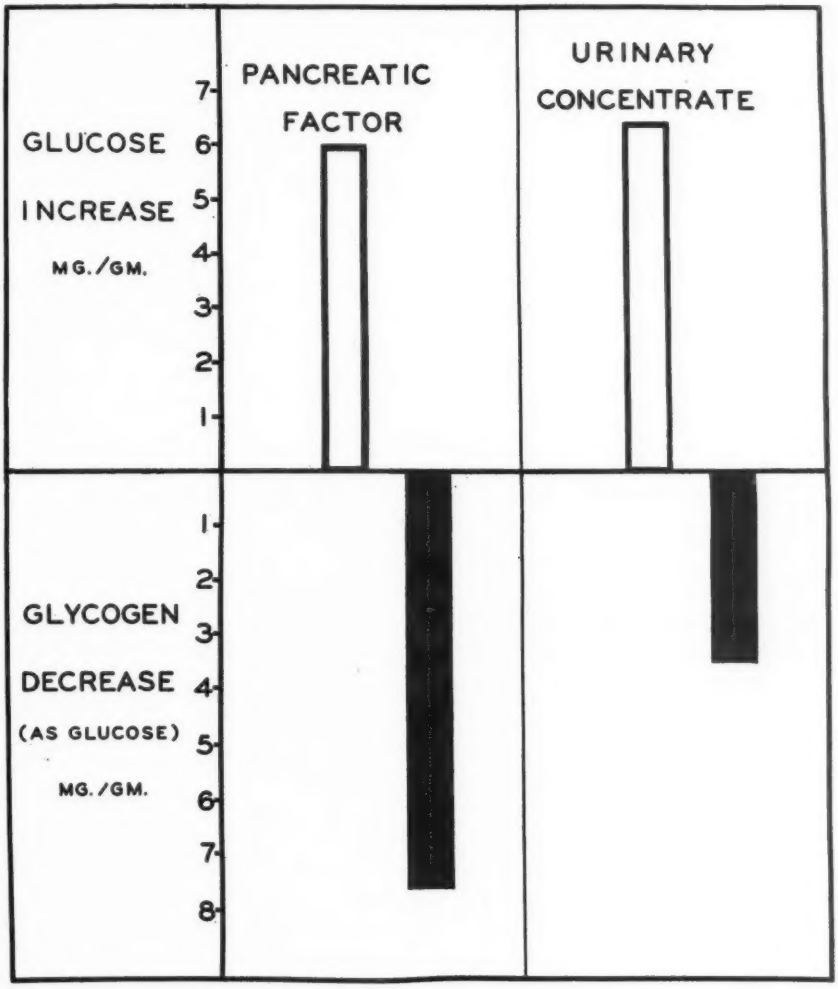

FIG. 3. Medium glucose and tissue glycogen changes after incubation of rabbit liver slices with urinary concentrate and pancreatic factor for 60 minutes. The bars represent the net changes obtained by subtracting the values found for untreated slices from the treated slices.

possibility that APU could act directly on liver glycogen, it was decided to test the effect of APU on glycogen in the absence of liver tissue. The incubation of APU (20 μg./ml.) at 37° C. for one hour in a solution containing 5 mg. glycogen and 0.06 mg. adenosine-5-phosphoric acid per ml. phosphate buffer (pH 7.4) did not result in glycogen disappearance.

TABLE 3. *In vitro* ACTION OF CALCIUM ION ON GLUCOSE OUTPUT BY LIVER SLICES AND HOMOGENATES

Tissue Preparation	Treatment	Dose μg./ml.	Glycogen Decrease[1]	Average	Glucose Increase[2]	Average
Slices	APU	20	—	—	8.9 7.0 8.9	8.3
	Calcium	24	—	—	4.8 3.6 3.9	4.1
Homogenate	APU	200	36.2 36.2	36.2	23.4 22.3	22.8
	APU	20	19.9	19.9	13.3 13.2	13.2
	Calcium	24	0.0 0.3	0.2	1.4 1.3	1.4

[1] Mg. glycogen (as glucose)/gm. tissue. Net decrease after subtracting decrease of untreated tissue.
[2] Mg. glucose/gm. tissue. Net increase after subtracting increase of untreated tissue.

DISCUSSION

The ability of the alcohol-precipitate from urine (APU) to stimulate glycogen breakdown by liver homogenates clearly distinguishes it from pancreatic glucagon which, under the experimental conditions described above, was inactive in this respect. Sutherland (1951) has shown that the glycogenolytic effect of epinephrine also requires intact cellular structure. It is concluded therefore that there is a glycogenolytic agent in the urine of normal human subjects which is neither epinephrine nor pancreatic glucagon. Another difference between glucagon and APU appeared when liver slices were treated with either factor. Whereas the additional glycogen disappearance induced by glucagon exceeded the increase in glucose output, under the action of APU the rise in glucose output was frequently accompanied either by little or no additional glycogen disappearance. It must be emphasized that the total glycogen disappearance found in APU-treated slices exceeded the total glucose release. The significance of this observation cannot be assessed in the light of the experiments presented above, and further experimentation is required to elucidate the mechanism of action of APU.

Extracts prepared from the urine of untreated diabetic patients induced active glycogenolysis in liver homogenates. The experiments reported above do not exclude the possibility that glucagon may be excreted in the urine, but they point to the necessity of distinguishing between the pancreatic factor and APU before arriving at any conclusions regarding the rate of urinary excretion of glucagon by healthy or diseased subjects.

Attempts to identify APU as one of several substances known to induce or favor glycogenolysis *in vitro* or to be hyperglycemic *in vivo* gave negative results for uric acid, growth hormone, pitressin, adenosine-5-phosphoric acid, and calcium ion.

SUMMARY

A crude concentrate or an alcohol-insoluble fraction derived from the urine of normal men and women has consistently been found to be hyperglycemic and to induce an increased release of glucose by rabbit liver slices *in vitro*. Unlike pancreatic glucagon, the urinary factor is very active in stimulating glycogen breakdown in liver homogenates. It is concluded that the glycogenolytic activity of urinary preparations is at least partially due to a factor other than pancreatic glucagon. Uric acid, growth hormone, pitressin, adenosine-5-phosphoric acid and calcium ion failed to stimulate glycogenolysis in rabbit liver homogenates.

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FURTHER STUDIES ON THE EFFECTS OF ADRENAL MEDULLARY HORMONES ON RENAL EXCRETION OF WATER AND ELECTROLYTES IN THE INTACT RAT[1]

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IT HAS been reported by several investigators that the subcutaneous administration of adrenaline causes increased water and chloride excretion during water diuresis in the rat (Stein and Wertheimer, 1944; Gaunt *et al.*, 1945; and Hays and Mathieson, 1945). Horres *et al.*, (1950) found 30 μg./100 gm. body weight of nor-epinephrine to be markedly diuretic, the same dosage of adrenaline was moderately diuretic and epinephrine at this dosage level had no effect on diuresis. These authors interpreted their results as indicating that adrenaline's diuretic effect was due to its content of nor-epinephrine.

After the report that low doses of epinephrine had no effect on water diuresis (Horres *et al.*, 1950; Eversole *et al.*, 1952), Drill and Bristol (1951) found that either epinephrine or nor-epinephrine in large doses (100 μg./100 gm. body weight) augmented urine flow during water diuresis. More recently Dexter and Stoner (1952) reported that l-epinephrine and d,l-nor-epinephrine improved the diuretic response in adrenalectomized or medullectomized rats. Such disparity pointed to the necessity for determining dose-response relationships for these two adrenal medullary hormones. Therefore, one purpose of this investigation was to determine whether the differences in the effect of the two adrenal medullary hormones on water and electrolyte excretion were qualitative or quantitative ones. Since the route of administration of hormones may influence responses, a

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[3] Acknowledgment is made to Dr. M. L. Tainter of Sterling-Winthrop Research Institute for the pure forms of the adrenal medullary hormones. An explanation of some terms used in this paper follows: Adrenaline—natural product containing epinephrine and nor-epinephrine; epinephrine—pure l-epinephrine bitartrate; nor-epinephrine—pure l-arterenol bitartrate monohydrate.

second purpose of the study was to determine whether intravenous administration would elicit responses comparable to those obtained after subcutaneous injection. In addition, the effects of nor-epinephrine on excretion of water and electrolytes were studied in animals under various degrees of hydration and under semi-chronic conditions where the hormone was injected daily.

Some of these observations were recorded in an earlier abstract (Giere and Eversole, 1953).

MATERIALS AND METHODS

Intact Sprague-Dawley rats weighing 200 ±10 gm. were used. They were fed Purina Chow and allowed water *ad libitum* unless otherwise noted; food was removed for the 15 to 18 hour period prior to administration of fluids and hormones. At the time of hormone injection the animals were placed in individual metabolism cages, given orally one dose of distilled water (3 ml./100 cm.^{2} of body surface, based on the formula of Benedict, 1938) warmed to body temperature. In the dose response studies the treated rats received aqueous hormone injected subcutaneously in the nape of the neck immediately after the water loading. Control animals received the solvent (water) subcutaneously. The volume injected was at least 0.05, but not more than 0.10 ml. The response to the hormone was determined by measuring, at one hour after injection, the urine volume (ml./100 gm. body weight) and urinary electrolyte concentration (m.eq./L). Since there is a day to day variation in the diuretic response to water, equal numbers of controls were run simultaneously with experimental animals. Each point on the figures represents the difference between the means of 12 control and 12 experimental cases.

Selecting a dosage of nor-epinephrine and a dosage of epinephrine which proved to be diuretic in the above experiment, the effects of these hormones were then tested separately in rats under the following conditions: (a) fifteen hour fast, water *ad libitum*, subcutaneous hormone injection but no water load; (b) fifteen hour food and water fast, subcutaneous hormone injection, but no water load placed on the animals; and (c) intravenous injection of nor-epinephrine into the saphenous vein of water-loaded rats. The intravenous injection was accomplished after exposing the vein by a small skin slit made when the area had been locally anesthetized with novocaine. Urine was collected for two hours.

In determining the effects of chronic administration of nor-epinephrine (20 μg. or 100 μg. in 0.05 ml. peanut oil per day) the rats were placed in individual cages over a glass funnel which separated the feces from the urine. Water intake and urine production were measured for twenty hours each day. During the four remaining hours the rats were permitted to feed but not to drink. Daily weight records indicated that body weights were maintained after the initial first day loss. Electrolytes were determined on two day samples for each rat. Parallel controls were injected subcutaneously with peanut oil but otherwise they were handled in the same manner as those receiving the hormone.

Chlorides were determined by the method of van Slyke and Sendroy (Peters and van Slyke, 1932). Sodium and potassium concentrations were determined by means of an internal standard flame photometer.

RESULTS

Effect of graded doses of adrenal medullary hormones on renal excretion of water and electrolytes.

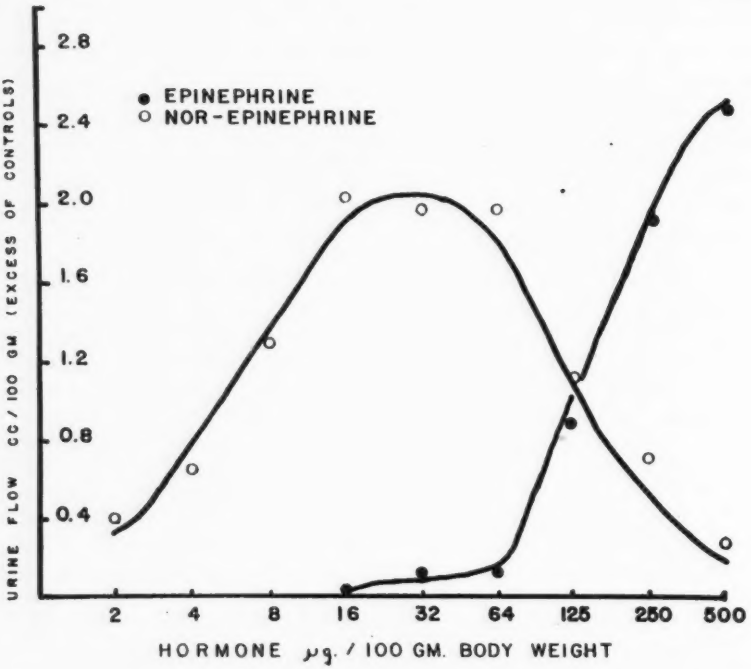

FIG. 1. Excretion of administered water after treatment with medullary hormones.

1. The data are presented graphically in Figure 1. When nor-epinphrine was injected in doses greater than 2 μg./100 gm. body weight, there was an increase in urine flow. Plotted as urine flow (in excess of control excretion) against the logarithm of the dosage, a rectilinear relationship was present between 2 and 16–32 μg. Dosages higher than this elicited a lesser effect upon diuresis. It was necessary to inject 64 μg. epinephrine to elicit an augmented urine flow. A rectilinear portion of the epinephrine curve was present between 32 and 250–500 μg. Higher dosages of epinephrine were not attempted because of the marked overdosage symptoms and subsequent sloughing of the skin at the site of injection. Minor overdosage symptoms were observed above 64 μg. epinephrine and only occasionally with the largest dosage of nor-epinephrine. The above observations were made at two times in the year, summer and winter, and although the absolute values were not identical, the rectilinear portion of the curves fell in the same dose range.

2. In regard to sodium and chloride excretion (Fig. 2), it was noted that there was no increase in sodium or chloride concentration in the urine until 32–64 μg. nor-epinephrine was administered, at which time near maximum

diuresis occurred. From this point, the sodium concentration rose sharply to a maximum at 250–500 μg. The chloride concentration also increased in dosages above 32-64 μg. and tended to, but did not, parallel the sodium.

Upon epinephrine administration, it was necessary to inject more than 64–126 μg. to elicit an appreciable natriuresis or chloruresis. Above that point both curves rose sharply and paralleled each other until the maximum dosage used here (500 μg.) was reached.

3. Potassium retention was observed (Fig. 3) with 16 μg. nor-epinehphrine and was maximal at 64 μg. Epinephrine did not cause potassium retention in the rat until 125 μg. dosages were administered; maximal effects were obtained with 250–500 μg./100 gm. body weight.

Effects of medullary hormones on non-hydrated rats. It can be seen in Table 1 that dosages of either hormone which were effective in the hydrated rat were also significantly diuretic, natriuretic and caused potassium retention in the non-hydrated animal permitted fluid the previous fifteen

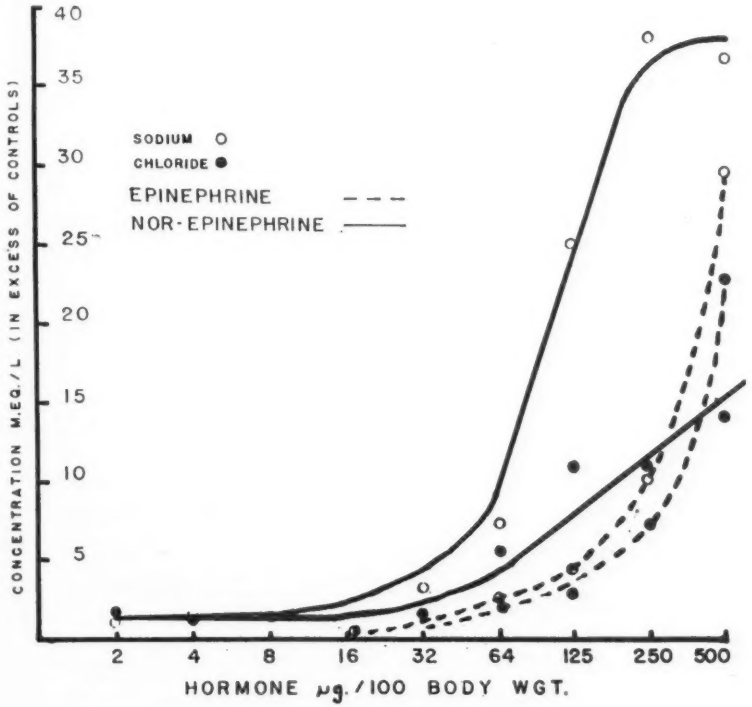

FIG. 2. Excretion of sodium and chloride in hydrated rats treated with medullary hormones.

TABLE 1. EFFECTS OF ADRENAL MEDULLARY HORMONES ON URINARY EXCRETION IN VARIOUSLY TREATED NON-HYDRATED RATS

Treatment	No. of cases	Urine vol. ml./100 gm. ±S.E.	Sodium*	Potassium*
(Food fast, but water *ad libitum* for previous 15 hours)				
Control	8	0.3±0.1	20.2±5.5	18.5±2.6
Epinephrine				
20 μg./100 gm.	8	0.4±0.1	14.7±3.6	9.2±3.7
P[1]		>.05	>.05	>.05
100 μg./100 gm.	8	1.4±0.3	72.0±2.4	3.4±0.7
P		<.005	<.001	<.001
Nor-epinephrine				
20 μg./100 gm.	8	1.6±0.1	42.8±2.7	3.6±0.6
P		<.001	<.001	<.001
100 μg./100 gm.	8	1.8±0.2	37.0±3.2	7.1±1.1
P		<.001	<.02	<.005
(Food and water fast for previous 15 hours)				
Control	8	0.04±0.03	—†	—†
Epinephrine				
20 μg./100 gm.	8	0.1 ±0.03		
P		>.05		
100 μg./100 gm.	8	0.3 ±0.1		
P		<.01		
Nor-epinephrine				
20 μg./100 gm.	8	1.1 ±0.2		
P		<.001		
100 μg./100 gm.	8	0.9 ±0.2		
P		<.005		

[1] "P" is calculated with respect to the control value for the appropriate group.
* In all tables electrolytes are given in terms of m. eq./L. and ±S.E. follows the mean.
† Urine volume too meager to analyze conveniently for electrolytes.

hours. The amounts of hormone effective were 100 μg./100 gm. of epinephrine or either 20 μg. or 100 μg. of nor-epinephrine/100 gm. These hormone dosages were also effective in increasing urine volume in water-starved rats.

Effects of intravenous nor-epinephrine on hydrated rats. It can be seen in Table 2 that 10 μg. aqueous nor-epinephrine administered intravenously to a 200 gm. rat elicited an increased urine flow and chloruresis and tended to alter, but not significantly, the concentration of sodium and potassium in the urine.

Semi-chronic administration of nor-epinephrine to intact rats. Rats which received 20 μg. nor-epinephrine in oil per day did not exhibit abnormal rates of water intake or urine and electrolyte excretion. Increasing the dosage to 100 μg. per day had no effect on water intake or urine output but tended to elicit urinary sodium and chloride concentration and potassium retention.

DISCUSSION

In the light of these investigations, the reason for the discrepancies reported concerning the effects of adrenal medullary hormones on water and electrolyte excretion in the rat now become obvious. The 33 μg. dose level

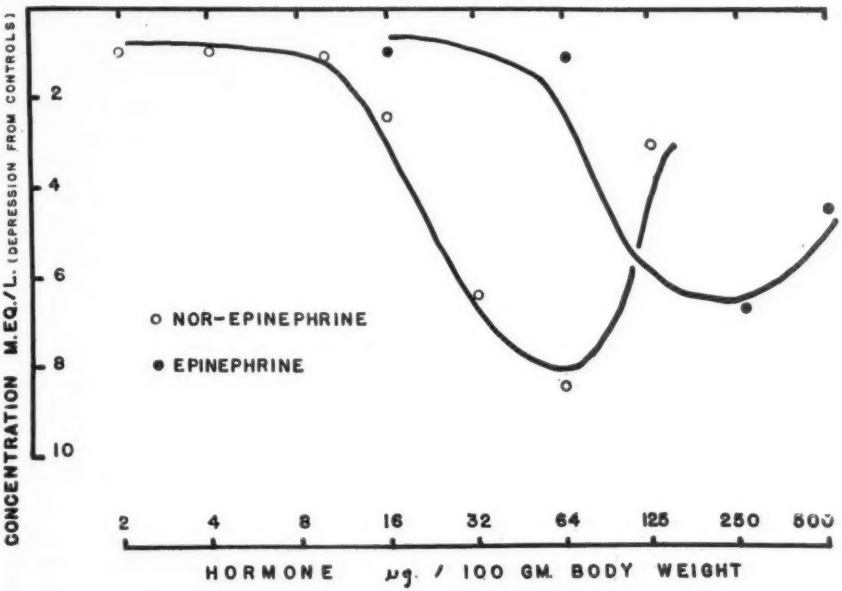

FIG. 3. Excretion of potassium in hydrated rats treated with medullary hormones.

of epinephrine previously used by our group is too low to elicit diuresis, natriuresis, chloruresis or potasium retention; however, the same dose of nor-epinephrine elicits a near maximal response for diuresis and potassium retention and does bring about some sodium and chloride excretion (Eversole, *et al.*, 1952). The results show further that at the dose level employed by others (Drill and Bristol, 1951), 100 μg./100 gm., epinephrine elicits responses similar to those of nor-epinephrine. A dosage of 100 μg. is in the rectilinear portion of the epinephrine curve and is on the negative slope of

TABLE 2. EFFECT OF INTRAVENOUS NOR-EPINEPHRINE[1] ON URINARY EXCRETION IN HYDRATED RATS

Treatment	No. of cases	Urine vol.[2] ml./100 gm. ±S.E.	Chloride	Sodium	Potassium
Control					
Water I.V.	6	2.8±0.3	5.4±0.6	2.7±0.6	7.3±1.3
Nor-Epinephrine					
1 μg.	6	3.3±0.3	6.4±1.9	2.7±0.8	6.9±0.7
P		>.05	>.05	>.05	>.05
10 μg.	5	4.4±0.4	8.8±1.4	4.2±1.2	6.3±0.6
P		<.01	<.05	>.05	>.05

[1] Administered under Novocaine local anesthesia.
[2] For 120 minutes.

the nor-epinephrine curve. The near identical diuretic properties of the two medullary hormones are therefore limited to approximately the 100 μg./100 gm. dose level in the rat.

Under the conditions of these experiments, it appears doubtful that increased water excretion is dependent upon a concomitant and disproportionate loss of salt. It has been noted that there is not an increased sodium or chloride concentration in the urine during the rectilinear phase of nor-epinephrine diuresis. Only when the rate of water diuresis decreases from the maximum increase is there a marked loss of sodium or chloride. It therefore appears that the effect of this medullary hormone on salt excretion is separate but not wholly independent of water diuresis.

From the data presented here, it would appear that the mechanisms for salt excretion which nor-epinephrine influences may be different from those influenced by epinephrine. This is based on the observation that the sodium and chloride excretion curves do not parallel each other with graded doses of nor-epinephrine, which is the case with epinephrine administration. The reason for such apparent difference is obscure.

In general, the findings indicate that the adrenal medullary hormones differ quantitatively in their effects on water and electrolyte excretion; nor-epinephrine being 4 to 16 times more effective than epinephrine as judged by the position of the rectilinear portion of the respective response curves. It has been our experience that these hormones are equally effective in water excretion, only at a dose similar to that used by Drill and Bristol (1951).

It appears that a dose of either hormone which elicits a diuresis, natriuresis or chloruresis in the acutely hydrated rat will elicit these responses regardless of the state of hydration or the route of administration. Although the local novocaine anesthesia used in preparing the rat for the intravenous administation of the aqueous hormone delayed the onset of the diuresis, the effect of the hormone was not entirely masked.

Daily administration of nor-epinephrine in oil (approximately 10 μg./100 gm./day) failed to elicit noteworthy changes in water intake or in urine and electrolyte output. Increasing the dosage to 50 μg./100 gm. /day tended to increase the urinary concentration of sodium and chloride and decrease the concentration of potassium. Such findings are in agreement with those of Duncan, *et al.* (1951) who reported that chronic administration of adrenal medullary hormones to the human causes increased urinary excretion of sodium and chloride, and diminished urinary excretion of potassium.

Information as to possible mechanisms of action of the adrenal medullary hormones on water and electrolyte metabolism was covered in a previous publication (Eversole, *et al.*, 1952).

SUMMARY

Based upon dose-response curves, the data presented here favor the view that the adrenal medullary hormones have a qualitatively similar action, but differ quantitatively, in enhancing water, sodium, and chloride output and potassium retention during water diuresis in the rat.

Hormone dosages which are effective in the above mentioned respects in hydrated rats are also effective in non-hydrated rats. The hormones also increase urine output in water-fasted rats.

Aqueous nor-epinephrine is diuretic and chloruretic when administered intravenously in a dose of 10 μg. per rat. Under the conditions of this experiment its natriuretic and potassium retaining properties were not demonstrated.

The intact rat allowed food and water *ad libitum* and injected daily for seven days with nor-epinephrine (50 μg./100 gm./day) failed to show changes in water intake and urine output but tended to excrete sodium and chloride and retain potassium.

Acknowledgment

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THE EFFECT OF GROWTH HORMONE ON MUSCLE POTASSIUM AND ON EXTRACELLULAR FLUID[1]

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INTRODUCTION

ALTHOUGH the nitrogen-retaining effect of growth hormone (GH) has been known for some time, it has only recently been demonstrated (Glafkides and Bennett, 1951) that potassium is also retained during GH administration. This finding is not unexpected since it is generally agreed that both potassium and nitrogen are retained simultaneously by growing tissues. A more surprising finding by these authors was the fact that in balance experiments sodium excretion is also reduced by GH, as it had not previously been recognized that GH had any effect upon sodium metabolism. As far as the authors are aware there are no data available concerning the effects of hypophysectomy or GH upon the muscle sodium, muscle potassium, or extracellular fluid. In view of the well known effects of target organ hormones, such as testosterone and adreno-cortical steroids, upon electrolyte metabolism, it would be surprising if hypophysectomy did not have some effect. In a further study of these phenomena we have carried out investigations to determine the distribution in the body of the retained sodium and potassium, to determine some of the conditions which alter the ability of the animal to retain these substances, and to eliminate the possibility that the observed effects are due to contamination of the GH by other pituitary hormones, or are mediated through certain other endocrine glands.

METHODS

The rats used in all these experiments were males of the Long-Evans strain varying in age from 50–90 days at sacrifice and were fed the usual stock diet[3] *ad libitum*. Hypophysectomy was performed by the parapharyngeal approach at 40–90 days of age and GH treatment was started from one day to two weeks later. The total time from opera-

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[3] The stock diet consisted of ground whole wheat 67.5%, casein 15.0%, whole milk powder 7.5%, NaCl 0.75% (with 0.01% KI), $CaCO_3$ 1.5%, hydrogenated vegetable oil 6.75%. To each kilogram of diet were added 3.5 gm. of Sardilene (fish oil concentrate containing 3000 USP units of vitamin A and 400 chick units of vitamin D per gram).

tion to autopsy varied from $1\frac{1}{2}$ to $2\frac{1}{2}$ weeks. The completeness of hypophysectomy was checked at autopsy by inspection of the hypophyseal fossa through a dissecting microscope. In general, two types of experiments were carried out: first, experiments in which were determined the effects of hypophysectomy and of GH administration upon (a) the concentration of sodium and potassium in body tissues and fluids and (b) the volume of plasma and extra-cellular fluid; second, experiments in which the effect of GH upon the sodium, potassium, chloride, and nitrogen balance of hypophysectomized rats was studied.

Sodium and potassium were determined by means of the *Perkin-Elmer flame photometer*, model 52A, using a lithium internal standard. Muscle samples of about 5 gms were cleaned and weighed, dried overnight at 60° C., reweighed and then ashed at 475° C. overnight. The ash was dissolved in 10 cc. of 1N HCl and then diluted to an appropriate volume for analysis by flame photometry. Diluted serum was analyzed directly without ashing. Two hours before autopsy, each animal was injected intra-peritoneally with a known, exactly-measured amount of NH_4SCN, usually about 6.5 mg. in 2 cc. The thiocyanate (SCN) concentration in serum was determined by the method of Bowler (1944) using the *Coleman Junior Spectrophotometer* at a wave length of 460 millimicrons. Although it is recognized that the SCN space is not ideal for measurement of the extracellular fluid volume, it was felt that it would be useful as an indication of the direction of change.

Plasma volume was determined by the Fe^{59}-labelled RBC dilution method. Labelled cells were obtained from a Long-Evans donor previously injected with Fe^{59}. The experimental animals were injected through the saphenous vein with 0.1 cc. of donor blood containing approximately 0.03 microcuries of Fe^{59}. After allowing six minutes for mixing, blood was drawn from the vena cava into a heparinized syringe. An aliquot of this blood was counted directly in a scintillation counter, while the hematocrit was determined on another aliquot. The total blood volume was calculated from the fraction of the injected activity recovered in the former sample. From the hematocrit and the total blood volume the plasma volume was calculated and the results are presented in terms of cc. of plasma per 100 gm. body weight.

For balance studies, animals were kept in individual metabolism cages over glass funnels containing perforated porcelain plates to separate out hair and feces. The food intake was constant for each rat during the period of the experiment. Collections were made every 24 hours, and the urine was analyzed for nitrogen by the micro-Kjeldahl method, for sodium and potassium by the flame photometer, and for chloride by a modification of the Volhard method (1932). Muscle from some animals was analyzed for its nitrogen content by the micro-Kjeldahl method and for potassium by flame photometry after wet-ashing in the Kjeldahl digestion mixture.

The GH and adrenocorticotropic hormone (ACTH) used were both furnished by Dr. C. H. Li of the University of California and were prepared by the methods of Li, Evans, and Simpson (1945, 1948). The growth hormone was assayed for antidiuretic activity by the method of Ham and Landis (1942) and found to be free of such activity at 500 μg., the largest single dose ever employed in these experiments. Parke, Davis *Pitressin*, Ciba testosterone propionate, and Squibb thyroxin were also used.

RESULTS

The thiocyanate space was determined in 7 series of rats. Each series consisted of a group of hypophysectomized controls, a group of normal controls, and a group of hypophysectomized animals treated with 1 mg. of

GH per day, intraperitoneally. In these experiments, the average thiocyanate space of the treated animals was elevated over that of the hypophysectomized controls, the increase being statistically significant in all instances. The data appear in Table 1. In four experiments identical with the above, the plasma volume was measured and found to be increased in all of the groups receiving GH, the elevation being statistically significant in 3 of the 4 groups. The data appear in Table 2.

Serum sodium and potasssium levels were measured in all animals where thiocyanate space determinations were carried out. Neither hypophysectomy nor GH administration altered the concentrations of sodium and potassium in the serum. The average serum sodium was 145.9, 146.9, and 145.2 meq. per liter for the normal, the hyophysectomized, and the hypophysectomized-GH treated animals, respectively. The average serum potassium was 4.98, 5.26 and 5.40 meq. per liter for the normal, the hypophysectomized and the hypophysectomized-GH treated animals, respectively.

Muscle samples from these animals were analyzed for their potassium, sodium, nitrogen and water content. Following hypophysectomy, the concentration of potassium per kg. of wet weight of muscle was lowered in all 11 series of animals, the decrease being statistically significant in 7. (The data on muscle potassium appears in Tables 3 and 4). Treatment with GH restored these levels to, or near, normal in 4 out of 5 series where GH

TABLE 1. EFFECT OF HYPOPHYSECTOMY AND GROWTH HORMONE (GH) ADMINISTRATION ON THE BODY THIOCYANATE SPACE

Series	Treatment	Body thiocyanate space expressed as per cent of body weight				
		Normal controls	Hypophysectomized	Difference	Treated hypophysectomized	Difference between hypophysectomized and treated hypophysectomized
XI	1 mg GH/day for 2 days (*Li 2265 AP*) (14)*	37.2±0.52†	36.9±0.64	−0.3 ($p = > .05$)	42.5±0.46	+5.6 ($p = < .01$)
IX	0.5 mg GH/day for 5 days, then 1.0 mg GH/day for 5 days (*Li 2265 AP*) (14)*	34.4±0.64	32.8±0.24	−1.6 ($p = > .05$)	37.9±0.83	+5.1 ($p = .02$)
I	1 mg GH/day for 10 days (*Li 10899*) (19)*	35.7±0.26	27.2±2.48	−8.5 ($p = < .01$)	40.6±0.76	+13.4 ($p = < .01$)
II	1 mg GH/day for 10 days (*Li 10899*) (15)*	35.2±0.80	36.6±0.71	+1.4 ($p = > .05$)	39.3±0.74	+2.7 ($p = < .02$)
III	1 mg GH/day for 10 days (*Li 10899*) (13)*	29.4±0.55	31.5±1.66	+2.1 ($p = > .05$)	35.2±1.55	+3.7 ($p = < .01$)
IV	1 mg GH/day for 10 days (*Li 1643 M*) (22)*	33.5±0.32	34.1±0.50	+0.6 ($p = > .05$)	37.8±0.81	+3.7 ($p = < .01$)
XIII	1 mg GH/day 11 days (*Li 2265 AP*) Gonadectomized (16)*	42.1±1.07	37.4±0.88	−4.7 ($p = < .01$)	44.8±1.16	+7.4 ($p = < .01$)

* Figure in parentheses indicates number of animals in the group.
† Standard deviation of the mean.

TABLE 2. THE EFFECT OF HYPOPHYSECTOMY, GH AND PITRESSIN ON THE PLASMA VOLUME

Series	Treatment	Plasma volume expressed as per cent of body weight		
		Hypophysectomized	Treated hypophysectomized	Difference
I	1 mg GH/day *Li 2265 AP* (14)*	2.62 ±0.045†	3.08 ±0.059	+0.46 ($p = < .01$)
II	1 mg GH/day *Li 2265 AP* (8)*	2.73 ±0.080	3.25 ±0.219	+0.52 ($p = < .02$)
III	1 mg GH/day *Li 2265 AP* (11)*	3.04 ±0.139	3.35 ±0.123	+0.31 ($p = > .05$)
IV	1 mg GH/day *Li 2265 AP* (12)*	2.82 ±0.074	3.15 ±0.038	+0.33 ($p = < .01$)
	4 mu Pitressin per day (14)*	2.82 ±0.074	2.90 ±0.101	+0.08 ($p = > .05$)

* Number in parentheses indicates number of animals in the group.
† Standard deviation of the mean.

was given for at least 10 days at a level of 1 mg. per day. However, treatment for 2 or 5 days resulted in no detectable rise in the muscle potassium concentration (Table 3, Series IX and XI). One exception to this should be noted. In Series IV of Table 3, although 1 mg. was given for 10 days, little alteration of muscle potassium was produced even though the SCN space did change as seen in Series IV, Table 1. This hormone, 1643 M, was not by any means as good a growth-promoting agent as the other two preparations, and therefore further experiments with it were not carried out. The tissue sodium level showed a tendency to be elevated following hypophysectomy and to be returned to normal by the administration of GH. These changes were not as consistent nor as marked, however, as the alterations in potassium concentration. In the two experiments where muscle nitrogen was determined, it was found to be unchanged following hypophysectomy, the normal controls averaging 35.3 mg./gm. of wet muscle with a range of 34.6–37.4, while the hypophysectomized animals averaged 35.4 mg./gm. of wet muscle with a range of 34.4–37.5. The water content of the muscle was calculated from the difference between wet and dry weights and is expressed as per cent of wet weight of muscle. Values of 75.9, 75.9, and 76.3% were observed for the normal controls, hypophysectomized and hypophysectomized-GH treated animals, respectively.

Certain control studies were carried out in order to test the possibility that contaminants of the GH preparation were responsible for the effects described above (see Table 4). The hormone was found to be free of anti-

diuretic activity at the dosage levels employed in these experiments, but, because of the known effects of posterior lobe preparations on water and salt balance, 3 different experiments were conducted in which *Pitressin* was administered at a level of 4 milliunits per day in 2 injections (2 milliunits is the minimum dose reliably detectable in our assays). In the first series, no elevation of SCN space or serum sodium was noted (Table 4, Series XVI). In a second experiment, no appreciable elevation of plasma volume was produced (Table 2). In a third experiment in which a balance study was carried out on 5 normal female rats a positive sodium balance was not produced.

Since testosterone causes both sodium and potassium retention, it was necessary to carry out experiments to rule out minimal ICSH contamination as the cause of the electrolyte changes. A group of 17 males was castrated 21 days and hypophysectomized 13 days before autospy. Half of the animals received 1 mg. of GH per day for 11 days. The changes in SCN space and K concentration were similar in every way to those of uncastrated, hypophysectomized animals (Tables 1 and 3, Series XIII). A second series of castrated-hypophysectomized males, in a balance study, showed definite retention of sodium and chloride during the administration of GH (Fig. 1). A group of hypophysectomized animals was given 5 mg./rat/day of testosterone propionate in sesame oil subcutaneously for 10

TABLE 3. THE EFFECT OF HYPOPHYSECTOMY AND GROWTH HORMONE (GH) ADMINISTRATION ON THE MUSCLE K CONCENTRATION

Series	Treatment	Normal controls	Muscle K concentration expressed as mEq/Kg of wet muscle			
			Hypophysectomized	Difference	Treated hypophysectomized	Difference between hypophysectomized and treated hypophysectomized
IX	0.5 mg GH/day for 5 days, then 1 mg/day for 5 days (*Li 2265 AP*) (16)*	98.2±1.61†	93.7±0.91	−4.5 ($p = .04$)	95.6±1.77	+1.9 ($p = > .05$)
XI	1 mg GH/day for 2 days (*Li 2265 AP*) (17)*	108.2±1.61	99.9±0.84	−8.3 ($p = < .01$)	100.3±0.73	+0.4 ($p = > .05$)
I	1 mg GH/day for 10 days (*Li 10899*) (19)*	95.5±3.29	95.2±2.59	−0.2 ($p = > .05$)	97.9±3.32	+2.7 ($p = .05$)
II	1 mg GH/day for 10 days (*Li 10899*) (15)*	109.5±2.37	100.1±2.13	−9.4 ($p = < .01$)	103.5±2.45	+3.4 ($p = .02$)
III	1 mg GH/day for 10 days (*Li 10899*) (13)*	105.0±1.08	101.1±2.25	−3.9 ($p = > .05$)	105.5±0.68	+4.4 ($p = > .05$)
IV	1 mg GH/day for 10 days (*Li 1643 M*) (22)*	104.5±3.30	96.6±2.15	−7.9 ($p = .02$)	94.7±1.20	−1.9 ($p = > .05$)
XIII	1 mg GH/day for 11 days (*Li 2265 AP*) Gonadectomized (17)*	112.4±0.89	107.9±1.31	−4.5 ($p = .02$)	115.8±2.50	+8.9 ($p = .02$)

* Number in parentheses indicates number of animals in the group.
† Standard deviation of the mean.

TABLE 4. CONTROL STUDIES

Series		Normal controls	Hypophysectomized	Difference	Treated hypophysectomized	Difference between hypophysectomized and treated hypophysectomized	Restricted food normals
VI (18)* 6 mg ACTH/day for 9 days	SCN Space % of B.W.	35.7± 1.26†	36.6± 0.70	− 0.9 (p=>.05)	33.2± 0.14	−3.4 (p=<.01)	
	Muscle K mEq./Kg. wet wt.	102.3± 1.85	91.0± 0.32	−11.3 (p=<.01)	90.4± 1.45	−0.6 (p=>.05)	
	Muscle Na mEq./Kg. wet wt.	31.2± 1.55	36.6± 0.65	+ 5.4 (p=<.01)	37.6± 1.53	+1.0 (p=>.05)	
XVI (19)* Pitressin 4 mu/day for 2 days	SCN Space % of B.W.	37.6± 0.66	37.2± 0.78	− 0.4 (p=>.05)	37.9± 1.76	+0.7 (p=>.05)	
	Serum Na mEq./l.	145.9± 0.63	143.5± 1.18	− 2.4 (p=>.05)	145.9± 0.63	+2.4 (p=>.05)	
XIV (21)* Restricted food, 20 days	Muscle K MEq./Kg. wet wt.	108.8± 1.20	102.7± 0.64	− 6.1 (p=<.01)			109.0± 1.08
XV (10)* Restricted food, 15 days	Muscle K mEq./Kg. wet wt.	114.1± 0.61					114.0± 0.74
XVII (14)* 5 mg. testosterone propionate per day for 9 days	SCN Space % of B.W.	34.6± 1.70	40.1± 1.18	+ 5.5 (p=.03)	37.4± 1.53	−2.4 (p=>.05)	
	Muscle K mEq./Kg. wet wt.	109.7± 1.29	102.2± 2.09	− 7.5 (p=.04)	102.9± 2.74	+0.7 (p=>.05)	
XVIII (16)* Thyroxine 3 γ for 2 days 5 γ for 8 days 7 γ for 4 days	SCN Space % of B.W.	34.2± 1.87	32.2± 0.29	− 2.0 (p=>.05)	30.7± 1.12	−1.5 (p=>.05)	
	Muscle K mEq./Kg. wet wt.	105.0± 0.78	100.1± 1.27	− 4.9 (p=<.01)	99.1± 1.14	−1.0 (p=>.05)	

* Number in parentheses indicates number of animals in the group.
† Standard deviation of the mean.

days. No alterations in SCN space or muscle potassium and sodium concentrations were observed when these animals were compared to hypophysectomized controls (Table 4, Series XVI).

A group of hypophysectomized animals was treated with 7 μg. of thyroxin per day and showed no alteration in either SCN space or K and Na levels in muscle as compared to their hypophysectomized controls (Table 4, Series XVII).

Although the GH preparation was considered to be free of ACTH, an experiment was carried out to rule out the possibility that ACTH contamination could be responsible for the effects of the GH preparation. Hypophysectomized rats were given 2 mg. of ACTH three times daily for 9 days (Table 4, Series VI). This treatment resulted in a fall of SCN space which was statistically significant, while the muscle potassium was not changed from the reduced level shown by the hypophysectomized control. Therefore, since ACTH altered the SCN space in the opposite direction from GH, and failed to elevate the muscle potassium concentration, it could hardly be responsible for the observed effects of the GH preparations. Another experiment bearing on this question has been reported by Stein, Bennett, Batts, and Li (1952) and shows that animals deprived of their adrenals and

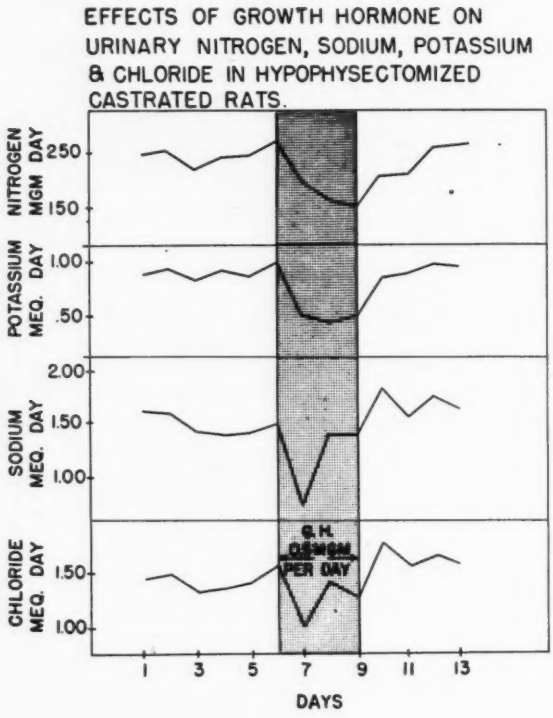

FIG. 1

maintained on aqueous cortical extract still retain sodium, chloride, and potassium during the administration of GH. Not only does this observation eliminate the possibility that ACTH contamination is involved, but also rules out the possibility that the GH itself exerts its effect through stimulation of the adrenal cortex as postulated by Selye (1951).

In an effort to rule out the possibility that reduction in food intake after hypophysectomy was responsible for the decrease in muscle K, two experiments were carried out in which normal animals were restricted in their food intake so that their weight curves paralleled those of the hypophysectomized animals. As can be seen in Table 4, Series XVI and XV, the animals on restricted food intake showed the same levels of muscle electrolyte as did the normal animals fed *ad libitum*, indicating that the reduction of muscle potassium after hypophysectomy is probably actually due to a hormone deficiency.

DISCUSSION

As noted above, the concentration of potassium in the muscle is elevated

above the level found in the hypophysectomized controls only when GH administration is continued for 10 days or more. This is in contrast to the finding that the expansion of the SCN space and plasma volume occurs within 2 days and is no greater even at 14 days. These observations can be correlated with the patterns of retention of sodium, chloride, and potassium seen in the balance studies in this paper and that of Stein, Bennett, Batts, and Li (1952). In these experiments all the retention of Na and Cl under the influence of GH occurred within 24–48 hours after the first injection and the excretion levels of these ions then returned to the control level. Therefore one would not expect the SCN space and plasma volume to continue to increase. However, the urinary potassium level continues to be low throughout the injection period, so that more total potassium has been retained at the end of 10 days of GH injection than at 2 or 3 days. The observation that about 10 days are required for the K concentration in muscle to be appreciably altered probably reflects the fact that the intracellular fluid volume is large and contains a high concentration of this ion and therefore a considerable amount of it must be added to this fluid compartment before the level will change measurably.

There is a general tendency in balance studies to assume that potassium is retained along with nitrogen in the ratio in which these substances are present in normal muscle. In making the calculation of this ratio, one determines the average daily excretions of N and K during the control period and subtracts from these values the average daily excretions during GH administration. This difference represents the average daily retention of N and K under the influence of GH. The ratio of retained N to retained K as calculated above is what we shall refer to subsequently as the N/K retention ratio.

In the balance study represented by Figure 1, the administration of GH to these hypophysectomized-gonadectomized males resulted in a N/K retention ratio of 2.42, which is markedly different from the N/K ratio of 4.17 found in the muscle of normal male rats of the same age. This difference in ratios indicates that these animals have retained extra potassium relative to nitrogen. Since it has already been shown that hypophysectomized animals exhibit a low muscle potassium but a normal muscle nitrogen, this observation suggests that when these animals are stimulated by GH to lay down extra tissue, they retain the substances necessary for this process in amounts that will tend to bring the muscle composition toward normal. From this point of view, one would expect the hypophysectomized animal with its low muscle potassium to retain extra potassium during GH administration, and one would predict that an animal which had an abnormally high muscle potassium would retain relatively less, *i.e.*, would show a N/K retention ratio higher than the ratio of nitrogen to potassium in muscle of

normal animals. To test this prediction, we calculated the N/K retention ratios during GH administration in the balance studies using adrenalectomized rats reported by Stein, Bennett, Batts, and Li (1952), because adrenalectomized animals are observed to show elevated muscle potassium concentrations (Cole, 1950). The three experiments reported by Stein, Bennett, Batts, and Li (1952) showed N/K retention ratios of 5.35, 3.65 and 6.48 as compared to a muscle ratio in normal female rats of the same age of 4.54. Moreover, these retention ratios were always higher than those seen in the groups of normal control females given GH at the same time (3.39 and 3.00). Although the data are admittedly scanty on these points, it suggests that a bit of caution is in order when the retention or loss of either K or N is calculated on the basis of data concerning the other.

It has been shown repeatedly, both in the balance studies of Stein, Bennett, Batts, and Li (1952) and in other work from this laboratory (Glafkides and Bennett, 1951) and (Whitney, 1952), that there is a sharp rise in sodium and chloride excretion following the cessation of GH treatment. This affords a possible explanation for the rather marked loss of weight in these animals at this time, since it probably represents loss of the extra volume of extracellular fluid. Moreover, during the course of these experiments it was noticed that the increase in weight during growth hormone treatment was often greatest in the first few days. Also, it has been shown (Lee, 1936) that the weight-loss following cessation of treatment amounts to some 70% of the weight gained if hormone has been given for 3 days, but if animals have been treated for 20 days, they lose only 20%–30% of the weight gained. This latter observation has been explained (Long, 1942) until now by assuming that protein is retained in the early stages in a labile form, perhaps in the liver, and only later is laid down in the muscles. However, the finding of an expanded extracellular fluid volume would seem to provide a different explanation, namely that more weight is gained in the beginning because, while tissue protein is being laid down continuously and equally throughout the injection period, the expansion of the extracellular fluid space occurs only in the first 1–2 days. Therefore, during three days of growth hormone treatment, a considerable percentage of extra weight would be gained due to fluid retention over and above the weight of tissue protein laid down. After cessation of treatment then, the loss of this fluid would result in the loss of a large percentage of the weight gained. After 20 days of treatment, however, the amount of fluid added to the body due to the expansion of the extracellular fluid in the first two days would contribute relatively little to the overall gain in weight, and by far the greater percentage of weight gained would be due to the laying down of actual tissue protein. Therefore, after cessation of

treatment, when the extra fluid is lost, the attendant weight loss would represent a much smaller percentage of the total gained previously.

SUMMARY

Because of the known ability of GH to cause Na, Cl, and K retention, the body thiocyanate space, plasma volume and muscle Na and K were measured in normal, hypophysectomized and GH treated-hypophysectomized animals. In seven series of 13–22 animals each, hypophysectomy produced no change in the thiocyanate space (normal = 34.2% of B.W., hypophysectomized = 33.2% of B.W.,) but administation of 1 mg of GH for 2–10 days elevated the SCN space to values as high as 42.5% of B.W. Moreover, in 4 series of 8–19 animals each, GH in the same dosage elevated the plasma volume 0.3–0.5% of the B.W. Plasma Na and K were unchanged. Muscle K concentration was lowered significantly following hypophysectomy in 10 of 11 series (average: NC 104.7 meq./kg. and hypophysectomized 98.6 meq./kg.). Administration of GH at 1 mg./day for at least 10 days restored the muscle K concentration toward normal (average—104.5). Muscle Na and N concentrations were unchanged.

To determine whether these effects are specific for GH, similar experiments were carried out using ACTH, *Pitressin*, thyroxine, and testosterone propionate. None of these agents produced the effects observed with GH. Moreover, gonadectomy did not alter the response to GH. Also restriction of the food intake of normal animals did not cause reduction of muscle K concentration as seen in the hypophysectomized controls. It is therefore concluded that GH causes an expansion of the extracellular fluid space, which explains the previously observed Na and Cl retention. Also GH plays some role in maintaining the muscle K.

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THE INFLUENCE OF THE PITUITARY-ADRENAL MECHANISM ON THE ACTION OF *LITHOSPERMUM RUDERALE*[1]

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SINCE the original report by Cranston (1945) numerous investigators have confirmed her observations that aqueous extracts of *Lithospermum ruderale* inhibit the estrous cycle in the female and androgen production in the male rodent. Associated with gross inhibition of reproductive function, decreases were also noted in the weights of the gonads, sex accessories, and thymus in rats (Cranston, 1945; Drasher, 1949) and interference with growth and development of immature mice (Cranston, 1945). Plunkett and Noble (1951) and Skelton and Grant (1951) further reported an increase in adrenal weight in adult male rats injected with aqueous extracts of lithospermum.

To date, no reports have been made on possible biochemical changes in the gonads, sex accessories, or adrenals associated with altered mass and function of these organs after the injection of extracts of *Lithospermum ruderale*.

Numerous investigators, since the report by Sayers *et al.* (1944), have correlated adrenal cortical activity with ascorbic acid and cholesterol levels within this gland in the rat. Adrenal enlargement was noted after injection of lithospermum in the present studies which had been under way at the time a similar observation was reported by Skelton and Grant (1951) and Plunkett and Noble (1951). Adrenal ascorbic acid and cholesterol determinations were therefore included in the present study as an indirect indicator of the degree of adrenal cortical secretory augmentation after lithospermum injection. Testicular ascorbic acid analyses were included to ascertain whether this constituent fluctuated with androgen synthesis by the testes as does its counterpart in the adrenal (cf. Sayers and Sayers,

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1948). Prostatic phosphatase determinations were made in an effort to correlate possible enzymatic changes with presumed testicular hormonal alteration, the latter evidenced by the decrease in weight of the prostate after lithospermum injections as noted by Cranston (1945), Skelton and Grant (1951) and in the present study.

The mode of action of lithospermum may be one of direct inhibition of the production of gonadotrophins (Cranston, 1945; Drasher and Zahl, 1946; and Cranston and Robinson, 1949), inactivation of circulating gonadotrophins (Zahl, 1948; Plunkett *et al.*, 1950), or some mechanism not yet characterized.

Adrenalectomized rats were included in the present experiments to determine the extent to which the adrenals might be concerned in the mechanism of action of *Lithospermum ruderale*. The present study reveals that a portion of the effects of lithospermum administered in the crude form required the presence of the adrenal glands.

MATERIALS AND METHODS

Adult male Sprague-Dawley rats were used throughout these experiments. Intact and adrenalectomized animals were fed *ad libitum* on Rockland rat chow pellets. Diets were supplemented twice weekly with fresh lettuce. Adrenalectomized rats received a 5% glucose–0.9% NaCl drinking solution immediately after adrenalectomy and for the duration of the experimental period. All animals were weighed and observed daily for signs of inanition (cf. Cranston, 1945).

The *Lithospermum ruderale* used for these experiments was obtained in a powder form derived from acetone precipitation of the aqueous extract of the ground plant material.[3] Ten grams of this material were stated to be equivalent to 0.5 lb. of roots, the administration of 10 mg./day to adult male rats reported to decrease the weight of the sex accessories 30–50%[4]

Lithospermum was administered subcutaneously either as an aqueous suspension or as a solution in phosphate buffer medium at pH 7.0.[5] The aqueous suspension was administered as 10, 15, or 30 mg. lithospermum in 0.5 or 1.0 ml. water to 6–8 rats per group. For the lithospermum in the phosphate buffer solution, injections of 6.0 mg./0.2 ml. were administered to 2–8 rats per group. Controls for each series were injected with equivalent volumes of the corresponding medium. The aqueous suspension was administered to intact animals daily for 12 days with autopsy on the 13th; in the case of the phosphate solution of lithospermum, the injection periods were 5, 12, or 33 days. Adre-

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[4] Dr. D. Beall, Ayerst, McKenna and Harrison, Ltd. Private communication, 1951.

[5] Gratitude is extended to Mr. Sanford Sigoloff for his suggestion of the use of phos phate buffer as a vehicle for lithospermum.

nalectomized rats were injected 1 day post-operatively and for 4 subsequent days with the phosphate solution of lithospermum only.

At autopsy, the adrenals, testes, and sex accessories were removed rapidly, secretions expressed from the sex accessories and all glands weighed. These organs were either prepared for analysis or frozen immediately on solid CO_2.

Ascorbic acid analyses were made by the method of Roe and Kuether (1943) and adrenal cholesterol content measured by the method of Schoenheimer and Sperry (1934). Sex accessory phosphatase activity was determined by the method of Shinowara, Jones, and Reinhard (1942).

RESULTS

Lithospermum injected as an aqueous suspension produced signs of inanition and toxicity when administered at 30 mg./day for 12 days. At this level a 20% decrease was noted in body weights (Table 1) and at autopsy poor absorption as well as signs of necrosis were noted at the areas of injection. Lower dose levels also inhibited weight gain. Lithospermum administered as a solution in phosphate buffer permitted an increase in body weights for the same injection period of 12 days (Table 1) and was apparently well tolerated by the animal. These rats seemed in good condition even after a 33 day daily injection of 6.0 mg. In the latter case however, weight gain did not equal that seen in appropriate controls.

In view of these obvious signs of inanition produced by the higher dose level of lithospermum in suspension, the adrenals were studied. Lithospermum as the aqueous suspension produced an adrenal weight increase which paralleled the dose level administered (Table 1, Fig. 1). It is apparent, however, (cf. Table 1, Fig. 1) that a certain degree of adrenal hyper-

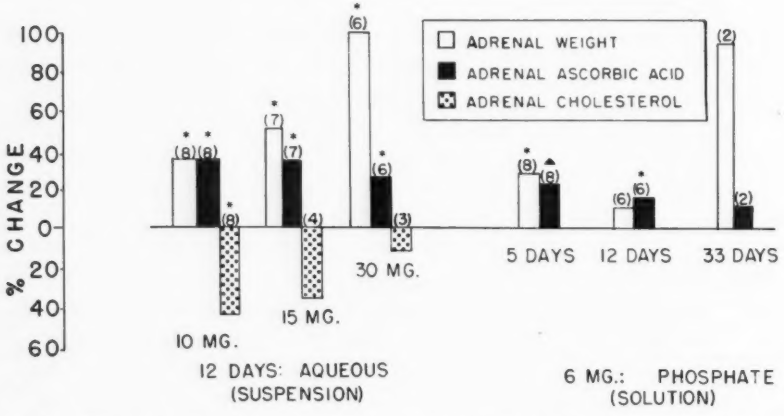

FIG. 1. Effect of *Lithospermum ruderale* on adrenal weight, ascorbic acid, and cholesterol concentration in intact rats. Figures in parentheses denote number of rats. * indicates a "P" value <0.01. Δ indicates a "P" value of <0.05.

TABLE 1. EFFECTS OF SUBCUTANEOUS ADMINISTRATION OF *Lithospermum ruderale* ON ORGAN AND BODY WEIGHTS OF ADULT MALE RATS

Treatment	Injection medium	No. rats	Days	Daily dose,	Body weight, gms.		Testes weight		Organ weights, gm./100 gm. B. W.		
					Orig.	Final	Gms.	Gms. 100 gm. B. W.	Sem. ves.	Prostate	Adrenals
INTACT											
Controls	Water	8	12	—	280 ± 13[1]	311 ± 22	3.11	1.00 ± 0.02	90.0 ± 11.8	56.1 ± 6.3	14.7 ± 0.4
Lithosperm.	Water	8	12	10	264 ± 10	259 ± 9	2.23	1.16 ± 0.03	75.0 ± 3.5	36.7 ± 2.2	18.0 ± 0.6
Lithosperm.	Water	7	12	15	302 ± 24	274 ± 15	2.40	1.14 ± 0.04	65.3 ± 4.0	40.5 ± 1.6	20.5 ± 0.8
Lithosperm.	Water	6	12	30	301 ± 43	237 ± 11	1.93	1.23 ± 0.03	44.8 ± 3.9	25.0 ± 3.7	31.6 ± 1.7
Controls	Phosphate Buffer	5	5	—	228 ± 21	235 ± 7	1.90	1.25 ± 0.08	75.0 ± 2.0	42.0 ± 4.5	13.9 ± 0.2
Lithosperm.	Phosphate Buffer	8	5	6	239 ± 10	236 ± 9	1.92	1.23 ± 0.04	55.0 ± 2.6	41.4 ± 8.0	17.6 ± 0.3
Lithosperm.	Phosphate Buffer	6	12	6	284 ± 19	313 ± 8	2.67	1.17 ± 0.04	70.4 ± 4.4	23.8 ± 4.4	15.3 ± 9.2
Controls	Phosphate Buffer	2	33	—	252 (247, 257)	312 (300, 324)	2.83	1.10 (0)	88.3 (86.2, 90.3)	81.7 (80.1, 83.3)	13.2 (13.0, 13.4)
Lithosperm.	Phosphate Buffer	2	33	6	210 (199, 221)	233 (224, 242)	1.88	1.19 (1.12, 1.26)	30.1 (21.3, 39.1)	31.6 (31.0, 32.2)	27.1 (26.0, 28.1)
ADRENALECTOMIZED											
Controls	Phosphate Buffer	3	5	—	242 (229–255)[2]	221 (218–224)	1.53	1.44 (1.35–1.53)	69.0 (67.2–70.1)	46.7 (46.2–47.1)	—
Lithosperm.	Phosphate Buffer	5	5	6	239 ± 7	206 ± 9	3.37	0.61 ± 0.03	51.0 ± 0.8	27.4 ± 3.7	—

[1] All errors are S.E.M.
[2] Ranges noted in parentheses.

trophy accompanied *Lithospermum ruderale* administration even under conditions not associated with inanition (12 day phosphate-buffer group).

Adrenal ascorbic acid levels increased significantly after the administration of all dose levels of lithospermum in suspension (Fig. 1). This finding was suggestive of the chronic reparative process in this tissue (Sayers, 1950) in which repletion may cause overshooting. In the case of rats injected with lithospermum in solution, adrenal ascorbic acid augmentation, though statistically significant at the 12-day treatment interval, was more moderate.

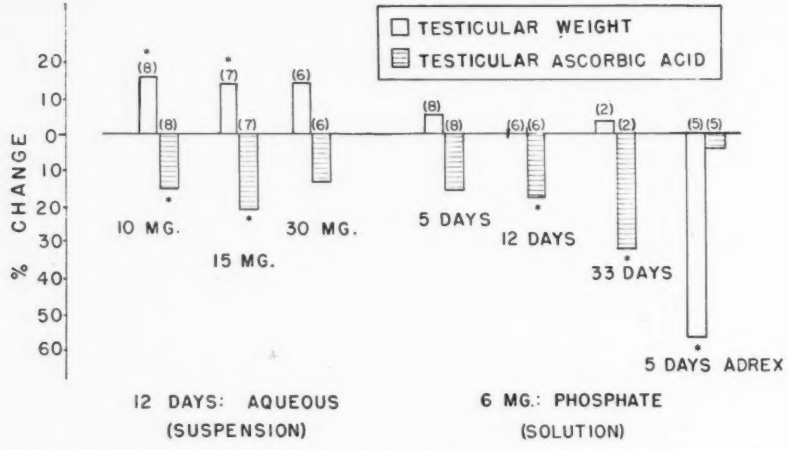

FIG. 2. Effect of *Lithospermum ruderale* on testicular weight and ascorbic acid concentration in intact and adrenalectomized rats. Figures in parentheses denote number of rats. * indicates a "P" value of <0.01. Weights are on a gm./100 gm. body weight basis.

Adrenal cholesterol concentration varied inversely with the dose levels of lithospermum injected as the suspension (Fig. 1). No data were collected from the groups receiving lithospermum in solution.

In view of the signs of adrenal implication in lithospermum action, it was decided to study the effects on the reproductive tract function in both intact and adrenalectomized rats. Testicular weight data are recorded for both groups in Table 1 and Figure 2. It will be observed in the intact group that with the exception of the 12-day lithospermum treatment in buffer all testicular weights suffered a decline from control values. That this decline did not keep pace with the general body weight loss in the suspension injected group is reflected in the apparent increase of relative testicular weights in these animals as compared with their controls (Fig. 2). Intact groups treated with lithospermum in buffer showed no significant change in relative testicular weights. The adrenalectomized animals, on the other

hand, showed a profound decrease in testicular weight following treatment with lithospermum.

A decrease was noted in testicular ascorbic acid levels in all treated groups except the adrenalectomized animals in which significant differences from control values were not observed (Fig. 2). The striking dependence of testicular ascorbic acid response to *Lithospermum ruderale* on adrenal function, together with the independence of testicular involution under the same conditions is of interest.

A study of the sex accessories was included for reflection of testicular androgen function. Weight losses were noted in the sex accessories in all rats administered lithospermum either as a suspension or solution (Table 1). This effect was not abolished by adrenalectomy. On the other hand, prostatic phosphatase levels, which in the intact group underwent even more striking decline than prostatic weight loss itself (Fig. 3), appeared to be unaltered by lithospermum treatment after adrenalectomy.

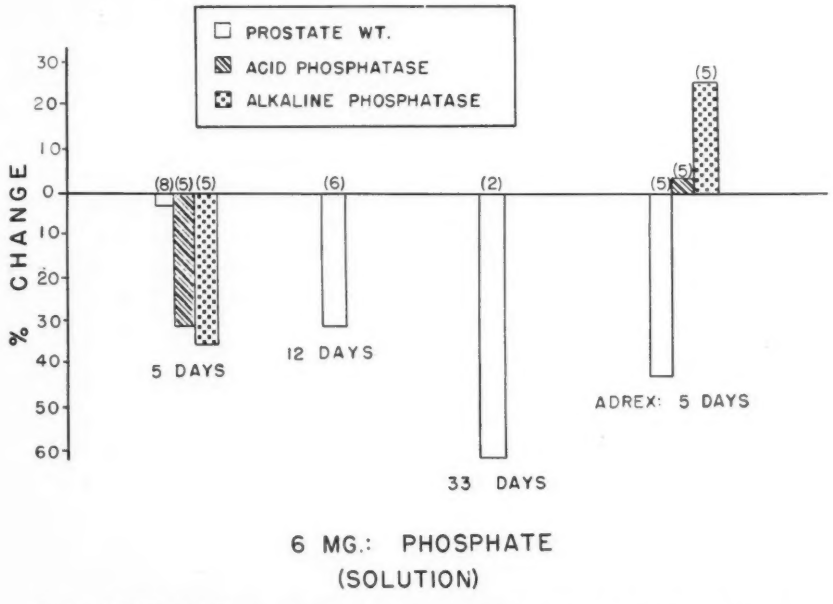

FIG. 3. Effect of *Lithospermum ruderale* on prostatic weight and acid and alkaline phosphatase concentration in intact and adrenalectomized rats. Figures in parentheses denote number of rats. Phosphatase values are in units/100 gm. body weight. Units of phosphatase activity have been based on the Huggins and Talalay (1945) definition: "10 units is the amount of enzyme which will liberate the colorimetric equivalent of 1 mg. of phenolphthalein from excess substrate in 1 hour at 37° C. under optimum conditions of pH."

DISCUSSION

The above data suggest that at least a portion of the effect of *Lithospermum ruderale* may require the presence of the adrenal gland. From the present experiments there seems little doubt that lithospermum in suspension, administered subcutaneously, acts as a stress in adult male rats. The increase in adrenal weights, the inanition and toxic effects noted were much like those observed by Skelton and Grant (1951). In addition a decrease in adrenal cholesterol of treated animals was noted in the present study.

On the other hand, lithospermum in solution appears to constitute less of a stress. The early signs of stress observed in animals injected five days (adrenal hypertrophy, lack of gain in body weights) are apparently partly compensated for by the animal in 12 days since at this period the adrenal weight increase was but slight and not statistically significant, nor were there signs of inanition or toxicity evidenced.

Nevertheless, the adrenal appears implicated in the mediation of effect of lithospermum in solution even in the absence of severe stress as noted above in presentation of the data on testicular mass and ascorbic acid. Thus, while testicular involution was profound in the adrenalectomized animals, the striking decline in testicular ascorbic acid (whether a reflection of tubular or interstitial tissue function) seen in intact treated animals could not be reproduced in the absence of the adrenals.

Since the report by Cranston (1945) numerous investigators have substantiated her data for decreased weights of the sex accessories as a reflection of failure of testicular androgenic function. Similarly in our experiments decreased weights for these organs were noted and this effect was not abolished in the adrenalectomized animal. On the other hand, the decreased acid and alkaline phosphatase values noted for the prostate in the intact *Lithospermum ruderale*-treated animal, were prevented by adrenalectomy.

Selye (1946) postulated a "shift" in pituitary hormone formation occurred under conditions of stress whereby adrenocorticotrophin (ACTH) production was increased at the expense of the gonadotrophins thus facilitating the defense of the organism. A portion of the mechanism of action of *Lithospermum ruderale* may be mediated by augmented pituitary-adrenal activity at the expense of gonadotrophin (Slusher, 1951) even in the groups not appearing to suffer severe stress (e.g. rats receiving lithospermum in solution).

The pituitary-adrenocortical augmentation seen in the treated animals may in turn be due to the presence of some adrenocorticotrophic hormone-like activity which is associated with the lithospermum. This may be present as a direct contaminant or as a non-specific activator of pituitary

adrenocorticotrophic function. Further study is indicated and awaits purification of the *Lithospermum ruderale*.

SUMMARY

At least a part of the mediation of effect of *Lithospermum ruderale* requires the presence of the adrenal gland. Testicular weight alterations, testicular ascorbic acid decreases, and decreased prostatic phosphatase levels observed in the intact adult male rat after treatment with lithospermum in solution, were not observed in similarly treated adrenalectomized rats. Furthermore, stress reactions such as adrenal hypertrophy, inanition, and toxicity resultant from administration of lithospermum in suspension were not obtained in rats administered lithospermum in solution for the same period of time.

Lithospermum ruderale may contain some adrenocorticotrophin-like factor present as a direct contaminant or as a non-specific activator of pituitary adrenocorticotrophic function. *Lithospermum ruderale* may only indirectly influence testicular metabolism by virtue of eliciting adrenocorticotrophin production and secretion by the anterior pituitary at the expense of production of gonadotrophins.

Acknowledgment

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ABSENCE OF STRESS-INDUCED AND "COMPENSATORY" ADRENAL HYPERTROPHY IN DOGS WITH HYPOTHALAMIC LESIONS[1]

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INTRODUCTION

IT HAS long been known that unilateral adrenalectomy is followed by compensatory hypertrophy of the remaining adrenal, and that this response is abolished by hypophysectomy (MacKay and MacKay, 1926; Collip, Selye and Thomson, 1933; Shumacker and Firor, 1934). It has been postulated that removal of one adrenal decreases the circulating level of adrenal corticoids, and that this lowered blood corticoid level leads to an increase in ACTH production. The increased ACTH production, in turn, results in contralateral adrenal hypertrophy.

Since certain hypothalamic lesions block the acute release of ACTH in response to a variety of stresses (Hume, 1953; Hume and Wittenstein, 1950; Porter, 1953; McCann, 1952). it was decided to investigate the effect of hypothalamic lesions on the development of compensatory hypertrophy after unilateral adrenalectomy.

MATERIALS AND METHODS

Adult mongrel dogs of both sexes weighing 10 to 14 kg. were used throughout. All females were in diestrus when used. The dogs were fed a diet of Ken-L-Biskit[4] supplemented by canned dog meat (Ken-L Ration[4]). Hypothalamic lesions were produced by a stereotaxic technique which will be described in detail elsewhere. Needles were implanted in the hypothalamus under x-ray control after filling the third ventricle with a radio-opaque dye, and current passed down these needles to produce the lesions. Hypophysectomies were performed through the transtemporal approach. Adrenalectomies were carried out through a lateral approach, resecting the last rib.

To determine the spontaneous variation of the differences between right and left adrenal weight normally seen in the dog, right and left adrenals from 30 autopsied dogs

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were weighed. Variation was expressed as per cent of left adrenal weight by the following formula:

$$\left[\frac{\text{right adrenal weight}}{\text{left adrenal weight}} - 1.00\right] \times 100 = \% \text{ variation in weight.}$$

The per cent variations were averaged and the standard deviation determined.

In addition, 25 animals were divided into the following groups:

Group 1—dogs with hypothalamic lesions. Twelve dogs were prepared with hypothalamic lesions in various locations. Following their recovery, they were twice tested for the presence or absence of an eosinopenic response to a standard surgical procedure and to forced immobilization.[5] One month from the time the lesion was made, left adrenalectomy was performed. Two weeks later the right adrenal was removed. Both adrenals were weighed.

Group 2—control dogs.

A. *Unstressed normal animals.* Six dogs were subjected to left adrenalectomy. Two weeks later the right adrenal was surgically removed. Both adrenals were weighed.

B. *Stressed normal animals.* Two dogs were subjected to abdominal laparotomy and allowed to recover. They were then twice tested for response to surgical trauma and immobilization in the same manner as the lesion animals. One month after the laparotomy, left adrenalectomy was performed, followed in two weeks by surgical removal of the right adrenal. Both adrenals were weighed.

C. *Hypophysectomized dogs.* Three dogs were hypophysectomized, twice tested for their eosinopenic response to surgical trauma and forced immobilization, and, one month after hypophysectomy, subjected to left adrenalectomy. In 2 of these animals, the right adrenal was removed surgically 2 weeks later.

Additional data were obtained from 2 dogs which had been hypophysectomized 2 and 5 months previously. The left adrenal was removed surgically from these animals and 2 weeks later the right adrenal was similarly removed.

All adrenals were dissected clean of fat and connective tissue as soon as they were removed, washed in saline, blotted once lightly on gauze, and weighed on a laboratory balance. In all cases, weights were converted to mg./kg. of body weight at the time of left adrenalectomy, and the difference between the right and left adrenal weight was expressed as a per cent change, using the above formula. All adrenals were fixed in 10% neutral formalin for at least 2 weeks, then sectioned for histologic examination. Frozen sections were examined under the polarizing microscope with and without acetone extraction, and under the regular microscope after staining with sudan black B, sudan IV, and the Schiff reagent (Dempsey and Wislocki, 1946). Permanent paraffin sections were stained by the technique of Klüver and Barrera (1953), and with hematoxylin and eosin.

The brains of all dogs with lesions were fixed *in situ* by perfusing 10% neutral formalin through the carotid arteries. The hypothalamus and pituitary were then removed in one piece, serially sectioned, and stained with eosin and Bullard's hematoxylin.

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[5] The standard surgical trauma used in these studies was a "standard operation" (Hume, 1953) which consists of incising the skin and vastus lateralis muscle for a distance of 14 cm. under pentothal induced ether anesthesis. The muscle edges are then retracted, and the muscle is freed up underneath. All bleeders are tied and the wound is closed in layers with fine cotton sutures. The procedure is timed to take exactly 30 minutes. Immobilization is carried out by restraining the dog in a special box which prohibits motion for a period of two hours.

RESULTS

A. Normal Dogs

Adrenal weights in the 30 autopsied dogs agreed with those obtained by Baker (1937), who measured adrenal weights in 1000 normal dogs. Surgically removed glands were slightly lighter than those removed at autopsy, though the difference was not statistically significant. In the present study, no significant weight difference between the adrenals from males and females was noted. There was very little difference in weight between the two sides, the mean ratio of right to left adrenal weight being 0.96. The standard deviation of right to left adrenal weight ratios was 6%. Therefore, assuming ±2 standard deviations as the limits of normal variation, the right adrenal could weigh from 16% less to 8% more than the left.

The surgically removed left adrenal glands of 6 normal dogs had a mean

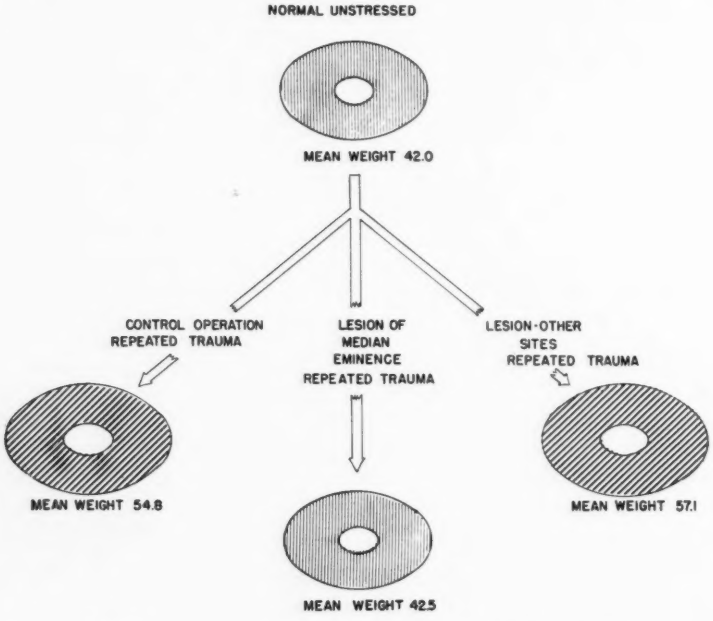
NORMAL UNSTRESSED
MEAN WEIGHT 42.0
CONTROL OPERATION REPEATED TRAUMA
LESION OF MEDIAN EMINENCE REPEATED TRAUMA
LESION-OTHER SITES REPEATED TRAUMA
MEAN WEIGHT 54.8
MEAN WEIGHT 42.5
MEAN WEIGHT 57.1
LEFT ADRENAL WEIGHT AFTER ONE MONTH
OF REPEATED ACUTE STRESS IN DOGS

FIG. 1. Hypertrophic response of the dog adrenal to repeated stress. All dogs were subjected to the same stresses over the course of one month, then the left adrenal was surgically removed and weighed. Figures are mean left adrenal weight in mg./kg.

weight of 42.0 mg/kg. of body weight (Fig. 1). The right adrenal glands surgically removed two weeks later had a mean weight of 50.4 mg./kg. This represents a mean increase in size of 19% and agrees well with the extent of the compensatory hypertrophy observed by others in the dog (Swinyard and Bruner, 1940). This increase is significant statistically when compared to the normal variation data ($P < 0.01$).

Histologic examination of these glands confirms the fact that hypertrophy has occurred. When compared to the left adrenal gland, the right adrenal shows an increase in cortical width, straightening of the cell cords, and finer vacuolization of the zona fasiculata.

In the 2 normal animals subjected to repeated trauma before adrenalectomy, the left adrenals were significantly heavier than those of the non-traumatized controls (mean weight 54.8 mg./kg.). The right adrenals, removed two weeks later, showed marked hypertrophy (mean weight 68.0

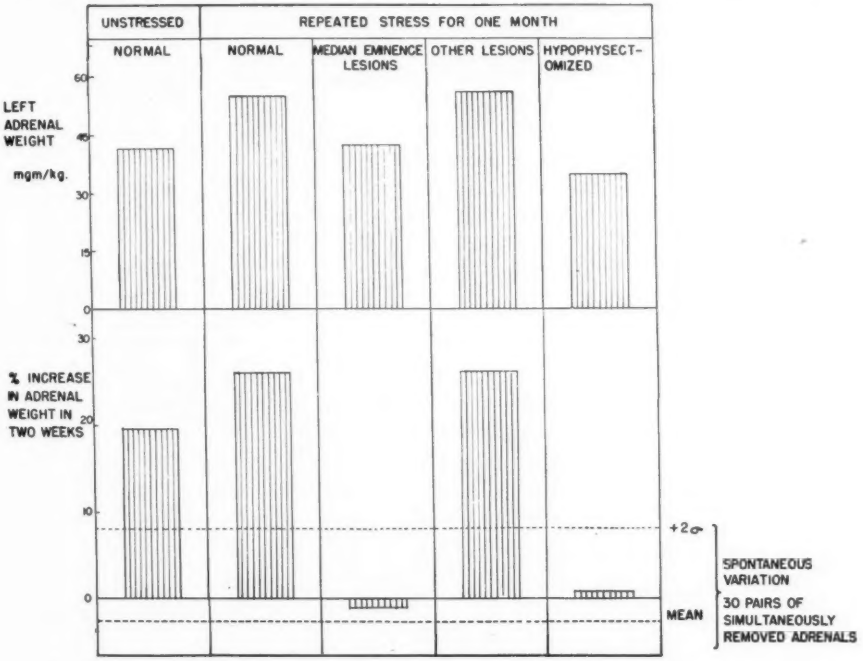

FIG. 2. Compensatory adrenocortical hypertrophy in dogs with hypothalamic lesions. The top columns represent mean weight of the left adrenal glands removed at surgery. The bottom columns show per cent increase in adrenal weight, and were computed by dividing the weight of the right adrenal, surgically removed two weeks after the left, by the weight of the left adrenal, subtracting 1.0, and multiplying by 100.

mg./kg.), and the percentage increase in size was the same as that of the unstressed normal animals (Figs. 2, 3).

B. Hypophysectomized Dogs

The mean weight of the left adrenal glands of the three dogs subjected unilateral adrenalectomy one month after hypophysectomy was 34.5 mg./kg. Histologically, these adrenals showed mild to marked diminution of the thickness of the cortex and large vacuoles in the cells. These results are similar to those of other investigators (Houssay, Biasotti, and Mazzocco, 1933; Houssay and Sammartino, 1933; Lane and de Bodo, 1952), who have found that histologic evidence of adrenal atrophy and decreased adrenal weight were usually present in the dog one month after hypophysectomy. It is of interest, in view of the work of Greep and Deane (1949), that the zone glomerulosa in these dogs was not atrophic. Indeed, it seemed slightly wider than normal, despite definite regressive changes in the fasciculata (Fig. 3).

The mean weight of right adrenals removed two weeks later was 34.9 mg./kg. Thus no increase in adrenal weight had occurred. Similarly, in the dogs operated three to five months after hypophysectomy, there was no significant increase in the adrenocortical weight after unilateral adrenalectomy. In these dogs, marked adrenal atrophy had occurred, the mean left and right adrenal weights being 26.8 and 28.2 mg./kg., respectively.

C. Dogs with Hypothalamic Lesions

Dogs with hypothalamic lesions fell into two categories. All animals in which the median eminence was essentially intact had normal responses, while all those with destruction of the median eminence showed abnormal findings.

1. *Hypothalamic Lesions Sparing the Median Eminence.* In 6 dogs the median eminence was normal. Two of these dogs had lesions in the pituitary, 2 had lesions in the area below the paraventricular nucleus, and 2, incomplete destruction of the mammillary bodies. The mean weight of the left adrenals in this group was 55.1 mg./kg. Histologically, they showed slight to moderate hypertrophy (Fig. 2). This weight is comparable to the mean weight of the left adrenals in the traumatized dogs, and significantly higher than the mean weight of the left adrenals in the non-traumatized controls. Thus, the mild hypertrophy in these lesion dogs can be explained by the stress of lesion production and repeated trauma.

The mean weight of the right adrenals removed two weeks later was 67.8 mg. Thus, definite compensatory hypertrophy had occurred, the increase in adrenal size being 23%. The histologic picture in these glands was that of definite, usually marked, hypertrophy (Fig. 3).

2. *Hypothalamic lesions destroying one-half or more of the median emi-*

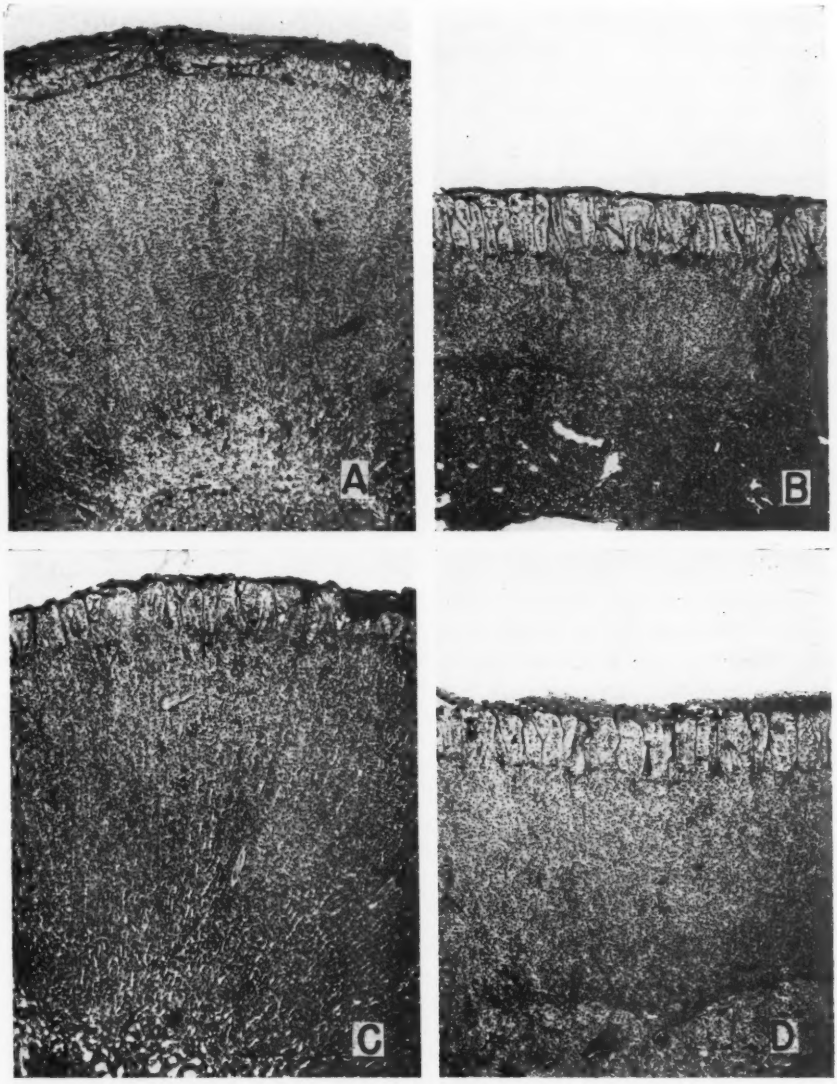

FIG. 3. Effect of unilateral adrenalectomy on the remaining adrenal in the dog. A) Right adrenal—normal dog. B) Right adrenal—hypophysectomized dog. C) Right adrenal—dog with incomplete destruction of mammillary bodies. D) Right adrenal—dog with destruction of median eminence. Hematoxylin and eosin stain, ×50. All dogs were subjected to left adrenalectomy two weeks before surgical removal of the illustrated right adrenals. In B, hypophysectomy was performed one month before left adrenalectomy and in C and D, hypothalamic lesions were produced one month before removal of the left adrenal.

nence. In 6 dogs, one-half or more of the median eminence was destroyed. The mean weight of the left adrenals in this group was 42.5 mg./kg. This is comparable to the left adrenal weights in the non-traumatized controls, but is greater than that found in the dog one month after hypophysectomy. Histologically, the adrenal appeared to be in the resting state, with large flakes of birefringent, sudanophilic material present throughout the fasciculata. There was, on the other hand, no evidence of adrenal atrophy like that found in the hypophysectomized dogs. Admittedly, the adrenocortical atrophy one month after hypophysectomy in the dog is not always marked. However, in other studies from this laboratory (Ganong and Hume, unpublished), lesion animals kept for periods as long as 8 months still show no significant adrenal atrophy in spite of continuously absent stress responses. McCann (1952) also finds no adrenal atrophy after lesions which abolish stress responses.

The mean weight of the right adrenals, removed two weeks later, was 42.3 mg./kg. This represents a weight change of -1%, and indicates complete absence of the compensatory hypertrophy which follows unilateral adrenalectomy in the normal dog and in all dogs with lesions sparing the median eminence. Microscopically, the right adrenals were similar to the left and no evidence of hypertrophy was present (Fig. 3).

It is interesting that there is a clear-cut correlation between the eosinopenic response to the stress of forced immobilization and standard surgical trauma, and the compensatory hypertrophy response. All of the animals with median eminence destruction, which showed no compensatory hypertrophy, also showed complete abolition of the eosinopenic response to operative trauma (mean 6-hour change was a 12% eosinophil rise) and to immobilization (4-hour change was a 2% rise). Animals which showed compensatory hypertrophy had mean eosinophil falls of 72% to surgical trauma and 58% to immobilization. These values are not significantly different from those found in the normal dog.

DISCUSSION

The lack of stress-induced and compensatory hypertrophy in lesion animals might in some way be related to pituitary damage secondary to destruction of the median eminence and the consequent, unavoidable, encroachment on the hypophyseal portal system. Unfortunately, a direct experimental approach to this point is difficult. However, the following observations suggest that pituitary damage is not the cause of the abnormal responses: 1) The pituitaries of all these animals were grossly normal, and histologically, although in some cases there were small areas of scar tissue involving particularly the anterior pole of the gland, there was always a large amount of normal pituitary tissue present. Trichrome and other spe-

cial stains were not used and no cell counts have been done, but microscopically, no obvious deviation from the normal was present. 2) These animals, as mentioned above, do not exhibit adrenocortical atrophy such as that found in the hypophysectomized animal, and regressive adrenal changes are absent. 3) The other target organs for pituitary hormones are variously involved, but they are not uniformly depressed. Thus, 3 of the 6 animals with median eminence lesions had depressed tyhoid function as measured by radioactive iodine uptake, while three had normal iodine uptakes. Similarly, on a weight basis alone, 4 of the animals with median eminence destruction had marked gonadal atrophy, but 2 had normal gonads. These observations will be reported in greater detail elsewhere (Ganong, Fredrickson and Hume, 1954). Their significance is not clear, but they do indicate that at least in some of these animals the pituitary is secreting thyrotrophic and gonadotrophic hormone at a normal or near normal rate.

Unfortunately, the literature regarding the effect of pituitary stalk section with interruption of portal-hypophyseal vessels is confusing, because in some species this produces gross pituitary infarction, and because many of the reports in the literature have not taken regrowth of these vessels into account (Harris and Jacobsohn, 1952; Barrnett and Greep, 1951; Uotila, 1940). On the other hand, transplantation of the anterior pituitary in a variety of animals does not abolish stress responses, even though it does usually lead to adrenocortical atrophy (Fortier, 1952; Fortier and Selye, 1949; Cheng, Sayers, Goodman and Swinyard, 1949; McDermott, Fry, Brobeck and Long, 1951).

The mechanism by which the removal of one adrenal leads to hypertrophy of the other is unknown, although many authors have assumed a decreased blood corticoid level as the initiating mechanism (Tepperman, Engel and Long, 1943; Sayers and Sayers, 1948). If this is true, the data in this study would suggest that some center in the hypothalamus is sensitive to *low* blood levels of circulating corticoids. It must be emphasized, however, that no direct evidence exists to prove a causal relation between a low blood corticoid level and increased pituitary ACTH production. Whether or not *high* levels of circulating adrenal steroids inhibit the pituitary through the hypothalamus or directly is under study at present, and preliminary results indicate that hydrocortisone induces the same degree of adrenal atrophy in normal dogs and in dogs with destruction of the median eminence (Ganong, unpublished).

Furthermore, in contrast to the pituitary transplant-animal which shows adrenocortical atrophy without complete loss of stress responsiveness, the median-eminence-lesion-animal has absent stress responses without significant adrenocortical atrophy. Apparently, therefore, the pituitary maintains a "basal" secretion of ACTH in the presence of hypothalamic lesions

abolishing compensatory and stress-induced adrenal hypertrophy, and this "basal" secretion can be depressed by high corticoid levels through some mechanism independent of the median eminence.

It is possible that low corticoid levels in the blood stimulate pituitary ACTH release through the median eminence, while high corticoid inhibition of secretion occurs elsewhere. Another possibility, however, is the hypothesis that stressful stimuli, including unilateral adrenalectomy, activate the hypothalamus through some unknown mechanism independent of the circulating corticoid titer. The actual amount of ACTH released from the pituitary is then determined by the balance between two independent variables, i. e. the magnitude of the hypothalamic stimulation and the extent of the corticoid inhibition. Such a hypothesis is inviting because it could explain why the animal can continue to produce ACTH in spite of high blood corticoid titers with continuing severe stress.

SUMMARY

A series of normal dogs and dogs with hypothalamic lesions have been subjected to comparable degrees of repeated acute stress and the effect of such stress on their adrenal histology and adrenal weight studied. In addition, a series of normal dogs and dogs with hypothalamic lesions have been subjected to left adrenalectomy, followed in two weeks by right adrenalectomy, and the weight and histologic appearance of the two glands compared.

Normal animals respond to repeated trauma with an increase in adrenal size and histologic evidence of adrenocortical hypertrophy. Hypothalamic lesions which do not involve the median eminence do not prevent this response, but when the median eminence is one-half or more destroyed this hypertrophy is absent.

Normal animals and animals with hypothalamic lesions sparing the median eminence show a 20% increase in right adrenal size two weeks after left adrenalectomy. On the other hand, no compensatory hypertrophy occurs in the dogs with lesions destroying the median eminence.

Dogs with destruction of one-half or more of the median eminence do not show adrenal atrophy even after prolonged periods of time. They are unlike hypophysectomized dogs in this respect.

The implications of these findings are discussed.

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HISTOCHEMICAL DEMONSTRATION OF DISULFIDE GROUPS IN THE NEUROHYPOPHYSIS UNDER NORMAL AND EXPERIMENTAL CONDITIONS[1]

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SPECIFIC histochemical methods for demonstrating protein-bound sulfhydryl (Barrnett and Seligman, 1952; Barrnett, 1953a) and disulfide groups (Barrnett and Seligman, 1954a) have been recently developed. The distribution of sulfhydryls (Barrnett, 1953a) and disulfides (Barrnett and Seligman, 1954a) was described in the tissues of albino rats and other animals. In the latter paper the possibility was raised that the sites of localization of disulfide containing protein hormones might be demonstrated in tissue sections if four criteria were satisfied. These would be: (1) that the hormone or its precursor contained disulfides; (2) that disulfides could be demonstrated by a specific method in tissues known to contain the hormone; (3) that available extracts of the hormone could be shown to be rendered insoluble by the same fixation as was applied to tissues and to stain by the same method for disulfides *in vitro*; (4) that physiological experiments would reveal alteration in the staining reaction in a predictable manner as the hormone is stored or released.

The antidiuretic hormone of the neurohypophysis should provide an excellent opportunity for testing this concept, since the physiological circumstances under which it is released have been widely investigated (see reviews of Verney, 1947; Waring and Landgrebe, 1950; and Pickford, 1952), and since also the active protein principle of the neurohypophysis contains a significant amount of disulfide present as cystine, but no cysteine (Irving and du Vigneaud, 1943).

The present communication deals with the histochemical demonstration of alcohol-soluble, disulfide containing material in the neurohypophysis of normal albino rats and other animals. In addition, the variation in the

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[2] The author wishes to acknowledge the technical assistance of Barbara Freeman and Nancy McAllaster.

amount of histochemically demonstrable material in the neurohypophysis of rats after dehydration and hydration was studied. A few experiments were also done on rats which had been stressed, had had the infundibular stalk sectioned, or had been hypophysectomized. Control experiments were performed to establish the specificity of the histochemical reaction, and *in vitro* experiments were performed on the precipitation and staining of fractions of neurohypophyseal extracts.[3] In addition, histological sections of the hypothalamus and infundibular stalk were stained by the method for sulfhydryls and disulfides since evidence, reviewed recently by Scharrer and Scharrer (1954), has indicated that the selectively stained neurosecretory material demonstrable in the hypothalamo-hypophyseal system of fibers as well as in the neurohypophysis may be related to neurohypophyseal hormones.

METHODS

Ninety-two albino rats were used in these studies, 54 of them for five sets of experiments and 38 as untreated controls. (1) Sixteen rats received no drinking water for 3 to 7 days and were allowed dried Purina dog chow *ad libitum* before they were killed. (2) Five rats which were dehydrated for 7 days were allowed water for 5 days before they were sacrificed. (3) Sixteen rats were hydrated by feeding them a diet consisting of one part Lactum[4] and nine parts tap water; no other food or drink was given, and after an initial period of a few days of adjustment to this diet, the animals drank a daily volume of fluid frequently equal to their body weight (avg. 221 gm.). After 7 to 20 days, these rats were killed. (4) Five rats were subjected to stress by the subcutaneous injection of 0.5 cc. of 10% formalin in the dorsum just caudal to the scapulae. These animals were killed within one hour after injection. (5) In a final group of 12 rats, the infundibular stalk was sectioned in 6 by a method already described (Greep and Barrnett, 1951), and 6 were hypophysectomized. After a period of at least 7 days postoperatively, these animals were killed.

The rats were killed by decapitation or, after anesthetization with ether, by perfusion of fixing fluids through the heart. The calvarium of each rat was opened and in approximately one-fifth of the unoperated experimental rats, and of the controls, the brain was removed with the hypophysis attached. The brain was trimmed to a block of tissue, consisting of the hypothalamus, infundibular stalk and attached pituitary gland and fixed. In the stalk-sectioned rats the pituitary gland and hypothalamus were fixed separately. In the hypophysectomized rats only the hypothalamus was fixed. In the remaining experimental rats and in the controls only the pituitary gland was saved and fixed. The pituitary glands either alone or combined with the hypothalamus were also obtained from 6 normal mice, 4 dogs, 2 cats, 2 monkeys (1 Cebus capucinus, 1 Macaca mulatta) and 1 armadillo.

The tissues from all the experimental and most of the control rats were fixed in Zenker's fluid, Zenker-formol, 10% formalin in saturated mercuric chloride, or in 80%

[3] Samples of Pituitrin, Pitressin and Pitocin were kindly supplied for this investigation by Dr. J. E. Gajewski, Parke-Davis Co., Detroit, Michigan.

[4] Lactum, a liquid formula for infant feedings, was kindly supplied by the Mead-Johnson Co., Evansville, Indiana.

ethyl alcohol containing 1% trichloracetic acid. A few of the tissues of the untreated control rats were also fixed in Bouin's solution.

The tissues were fixed for 24 hours, and washed in either 80% or 90% alcohol overnight. Those fixed in solutions containing mercuric chloride were treated with Lugol's solution. The tissues were then dehydrated and embedded in paraffin in the routine manner. The blocks of the hypothalamus, stalk and attached pituitary gland were serially cut in the sagittal plane in 5 or 10 μ sections, whereas the pituitary glands alone were cut in the frontal plane in sections of the same thickness.

Some of the sections of tissue fixed in alcohol-trichloracetic acid were stained for protein-bound sulfhydryl groups alone (Barrnett and Seligman, 1952) and some by the method for combined protein-bound sulfhydryls and disulfides (Barrnett and Seligman, 1954a). In the former method, sulfhydryl groups were reacted with the specific reagent, 2,2′-dihydroxy-6,6′-dinaphthyl disulfide (DDD)[5] and a red or blue color was developed at the sites of reaction with tetrazotized diorthoanisidine.[5] In the latter method, after reduction of disulfides with thioglycolate or other reducing agents, the sulhydryl reaction was applied. This procedure demonstrates the total sulhydryl content (free and those derived from reduction of disulfides). Consequently, the differences in intensity and distribution of staining reaction between sections treated by the two methods reveal the sites of disulfides.

The tissues subjected to the other fixatives were stained by the combined method for sulfhydryls and disulfides (Barrnett and Seligman, 1954a). It should be pointed out that it is not possible on the basis of the histochemical reaction alone to determine whether sulfhydryls, disulfides, or both, are contained in a protein fixed in a solution containing either potassium dichromate or mercuric chloride. Presumably the dichromate could oxidize the sulfhydryls to disulfides and the mercuric salt could form mercaptides with sulfhydryls. During reduction of the naturally occurring disulfides, the disulfides produced by oxidation with dichromate would be reduced and the mercaptides would be reversed by the sulfhydryl containing reducing agent and the OH^- concentration required for reduction. However, the possibility also exists that the protein fixed by the reagents might contain disulfides but no sulfhydryls. Occasionally, sections cut at 5 μ were also stained by Gomori's (1941) chrome-alum-hematoxylin and phloxine method, which, as Bargmann (1949a) and others have shown, is a selective histological method for the demonstration of neurosecretory material. In this paper, the staining produced as a result of the chrome-alum-hematoxylin method shall be referred to as selective staining, and that of the sulfhydryl and disulfide method shall be referred to as the histochemical method.

In the *in vitro* experiments, 5 cc. aliquots of *Pituitrin*, *Pitressin* and *Pitocin*[3] were used as samples of neurohypophyseal protein hormones. These fractions were precipitated by the addition of non-alcoholic fixatives. The precipitates were collected, either on filter paper or in centrifuge tubes, washed repeatedly and carried through each step used in the preparation of tissue sections and histochemical staining procedure for sulfhydryl and disulfide groups, except paraffin embedding. The precipitates were exposed to the various reagents for the same lengths of time required in the fixation, preparation and staining of the tissue sections. The specificity of the reactions of both the tissue sections and the *in vitro* precipitates for free sulfhydryls, as well as for sulfhydryl derived from the reduction of disulfides, was established by methods described elsewhere (Barrnett, 1953a; Barrnett and Seligman, 1954a).

[5] Schwarz Laboratories Inc., 202 E. 48th St., N. Y.

RESULTS

Histochemical and selective staining of the neurohypophysis of normal animals. Sections through rat infundibular process, fixed in alcohol-trichloracetic acid and stained for sulfhydryl groups were weakly reactive. This sparse reaction was distributed in the pituicytes and stromal cells and in the nerve fibers ramifying throughout the gland (Fig. 1). Similarly fixed sections stained for sulfhydryl and disulfide groups showed a slight over-all intensification of the reaction without any apparent change in the distribution (Fig. 2). This reaction was in marked contrast to the staining of colloid material located in the residual cleft of the pituitary, between the anterior and intermediate lobes, which was intensely reactive by the method for sulfhydryl and disulfide but not for sulfhydryl alone (Fig. 2). No neurosecretory material was demonstrable in the alcohol fixed glands by the selective histological method (chrome-alum-hematoxylin).

The infundibular process in sections of rat pituitary glands, which were fixed by the other methods and stained for sulfhydryls and disulfides, showed a greater intensity of staining reaction (Fig. 3). This was due not only to an over-all increase in intensity of staining of the previously mentioned structures, but also to the presence of large amounts of a new reactive material. Throughout the infundibular process numerous globules or vesicular bodies were found to be moderately stained. These colloid-like structures varied in size from small droplets to large vesicles (Figs. 3, 9, 18). They appeared rather evenly distributed throughout the infundibular process but frequently the central portion seemed less intensely populated with them. Some of the reactive material occurred in the connective tissue between the infundibular process and intermediate lobes, but was never found within the intermediate or anterior lobes. Frequently, this material was localized around the blood vessels of the infundibular process (Fig. 9). There was a slight but noticeable variation in the infundibular process of the control group as to the amount of reactive material present and the intensity with which it stained.

It was not possible to indicate with complete assurance whether or not some of the positively stained material was in the pituicytes. This was in part due to the thickness of the sections, a necessity created by the lack of intensity of staining in sections cut at less than 10 μ. In many instances, the number of stained globules was so great as to obscure completely the structural details of the gland (Fig. 3). Frequently, isolated reactive material was seen which had no apparent relationship to the pituicytes whatsoever, but rather appeared in tissue spaces surrounded by pituicytes and nerve fibers (Fig. 14). In some instances pituicytes were seen completely surrounded by reactive material. It was also not possible with the present

material to decide whether or not the reactive material occurred within nerve fibers.

The material which was reactive by the histochemical method appeared to be the same as the material which was stained by the selective histological method (Fig. 4). This was deduced from the distribution of the material stained by the two methods in consecutive 5 μ and 10 μ sections, the former stained with chrome-alum-hematoxylin and the latter for sulfhydryl and disulfide. The colloid droplets could be compared in this manner but not the smaller globules or granules of reactive material. In all instances, the staining with chrome-alum hematoxylin was more intense than with the sulfhydryl-disulfide method.

With the histochemical method, tissues fixed in sublimate formol stained most intensely, whereas those fixed in Bouin's fluid, stained least intensely.

EXPLANATION OF PLATE 1

All figures are of sections of rat infundibular process of the neurohypophysis. Magnification is ×350 except Figure 3 which is ×600.

FIG. 1. Normal rat. Alcohol-trichloracetic acid fixation. The cytoplasm of pituicytes and the nerve fibers stain for sulfhydryls. No neurosecretory material is preserved.

FIG. 2. Normal rat. Alcohol-trichloracetic acid fixation. The sulfhydryl and disulfide reaction is more intense than the sulfhydryl reaction alone. A portion of the intermediate lobe of the pituitary is included and the colloid of the residual cleft (bottom) which is intensely reactive for sulfhydryl and disulfide, but not for sulfhydryl alone.

FIG. 3. Normal rat. Formol-sublimate fixation. The sulfhydryl and disulfide reaction stains numerous globules and vesicular accumulations of neurosecretory material which are preserved.

FIG. 4. Normal rat. Zenker's fixation. Chrome-alum hematoxylin stains a morphologically similar material to that indicated in Figure 3.

FIG. 5. Dehydrated rat (5 days). Zenker's fixation. The material which stains with chrome-alum hematoxylin is virtually absent. In the center of the photomicrograph there is one droplet of neurosecretory material. The center of this droplet stains differently than the periphery. The remaining stained structures are nuclei of pituicytes and stromal cells.

FIG. 6. Dehydrated rat (7 days). Sublimate-formol fixation. The neurosecretory material which stains with the sulfhydryl-disulfide reaction is not present. Erythrocytes in the blood vessels stain for sulfhydryls and disulfides after this fixation but not after alcohol-trichloracetic acid.

FIG. 7. Dehydrated rat (5 days). Zenker's fixation. The staining of the neurohypophysis for sulfhydryl and disulfide is similar to that in Figure 6. Colloid of the residual cleft is as intensely stained as after fixation in alcohol-trichloracetic acid.

FIG. 8. Dehydrated rat (3 days). Zenker's fixation. Not all the sulfhydryl and disulfide reactive material has been depleted. Hollowed out vesicles, free of stained material, can be made out.

FIG. 9. Dehydrated rat (5 days) which was allowed water (5 days). Sublimate-formol fixation. The sulfhydryl and disulfide reactive material is present in normal amounts and stains intensely. Figure shows the relationship of this material to a blood vessel in the infundibular process.

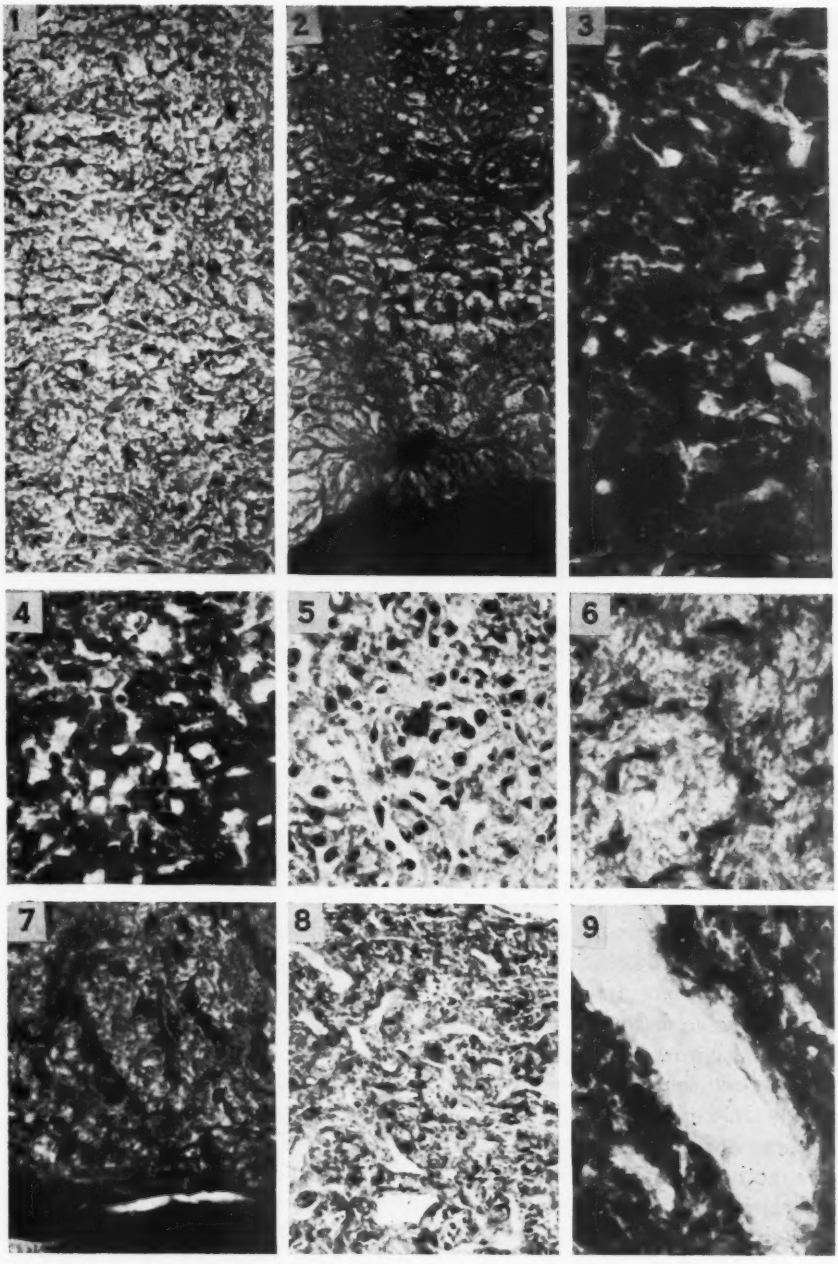

PLATE 1

The tissues fixed in Bouin's fluid also gave poorer results for the selective staining with chrome-alum-hematoxylin. In some instances, especially after prolonged washing of tissues in 80% alcohol, the globular material in Bouin-fixed sections did not stain for sulfhydryls and disulfides, but reacted strongly with chrome-alum-hematoxylin.

Similar material, reactive to the above selective and histochemical methods, was demonstrable in the infundibular processes of mouse, dog, cat, monkeys and armadillo. However, there was frequently less of the stainable substance in some of these specimens. This was possibly due to the fact that none of these hypophyses was perfused with the fixatives, as in the rats, and that there was a greater time lapse before the glands were fixed. In some animals, especially in the dog, the colloid droplets were much larger than seen in the rat. The larger droplets in the dog always stained less intensely for sulfhydryls and disulfides than the smaller ones found in the rat.

In the infundibular stalk and median eminence of the rat, there were only a few, randomly dispersed, colloid droplets demonstrable by either the histochemical (Fig. 12) or the selective histological method. Some sections of the stalk, indeed, contained no stained material.

Effects of dehydration, hydration, stress and stalk-section. Sections of neurohypophyses of rats which were dehydrated for 3 to 7 days showed marked changes in the content of material stained by the sulfhydryl and disulfide method (Figs. 6, 7) and by the selective method (Fig. 5). These changes were most pronounced in neurohypophyses of the animals dehydrated longest. In most specimens stained by the histochemical method, the depletion of reactive material was complete. The appearance of these sections (Figs. 6, 7) resembled that of sections fixed in alcohol-trichloracetic acid and stained for sulfhydryls and disulfides (Fig. 2), whereas the material had actually been fixed in Zenker's fluid or sublimate formol which are better protein precipitants and preserve the reactive material as indicated in the studies on the control neurohypophyses and by the staining of sulfhydryl and disulfide groups of erythrocytes in the dehydrated specimens. In a few specimens, however, a variable but small amount of reactive material remained after this degree of dehydration (Fig. 8). This material was usually present in the form of large droplets rather than as small granules. Beside the over-all depletion of sulfhydryl and disulfide reactive material, the large colloid droplets presented a hollowed-out appearance. In sections of infundibular process of dehydrated rats stained by the selective method, the centers of the rare, colloid droplets stained with phloxine rather than hematoxylin. The amount of reactive material in the stalk remained sparse. The infundibular process of each of the five rats which were first dehydrated and then rehydrated appeared similar to that of untreated controls (Fig. 9).

Sections through the infundibular processes of rats, which drank at least 5 to 6 times the normal amount of fluid daily, showed a variable response. In a few cases, there was an over-all increase in the amount of histochemically or selectively stained material, in comparison to untreated control specimens (Fig. 13). However, some of the infundibular processes appeared to contain no more reactive material than the controls and some appeared to contain less (Fig. 14). The infundibular stalks did not contain an increased amount of reactive material even in those specimens in which the infundibular process did.

Only the infundibular processes of the stressed rats were examined and the earliest specimens were taken 10 minutes after the subcutaneous injection of formalin. In these, there was an obvious decrease in the amounts of both histochemically and selectively reactive material (Fig. 15), but the degree was not as great as in any of the dehydrated specimens. Collection of stained material about dilated capillaries was common and sometimes the stained material was seen within capillaries. The phenomenon noted in dehydrated rats, in which the colloid droplets appeared hollowed-out, was also observed in these animals. By one hour, there had been no reaccumulation of reactive material in the infundibular process and little, if any, further loss occurred.

None of the 6 stalk-sectioned rats displayed severe diabetes insipidus, but all of them drank more water (average, 35 to 52 ml/day) than controls (average, 26–29 ml/day). In sections through the infundibular process of two of these animals, no sulfhydryl-disulfide reactive material was demonstrable (Fig. 16). In the remaining four, the amount of reactive material and the intensity with which it stained were diminished in comparison with controls (Fig. 17).

Hypothalamus. The hypothalamus of untreated rats and dogs, stalk-sectioned rats and hypophysectomized rats was investigated for sulfhydryls and disulfides.

The results of the histochemical staining of rat hypothalamus were not clear cut because of the intense sulfhydryl and disulfide reactions of nerve fibers which obscured the clear recognition of colloidal droplets. However, in the dog, where colloid aggregates are larger than in the rat, it was found that these were present in the supraoptico-hypophyseal tract and stained weakly for sulfhydryl and disulfide. Two further observations on the staining of the hypothalamus are pertinent. The cytoplasm of some of the neurons of the supraoptic nucleus in the dog and rat was more intensely reactive for sulfhydryls and disulfides than that of other hypothalamic neurons (Fig. 10). This reaction was not confined to the intracellular droplets but was rather evenly distributed throughout the cytoplasm. Secondly, sections of hypothalamus of 2 stalk-sectioned rats showed that the region of the median eminence and the proximal stump of the infundibular stalk

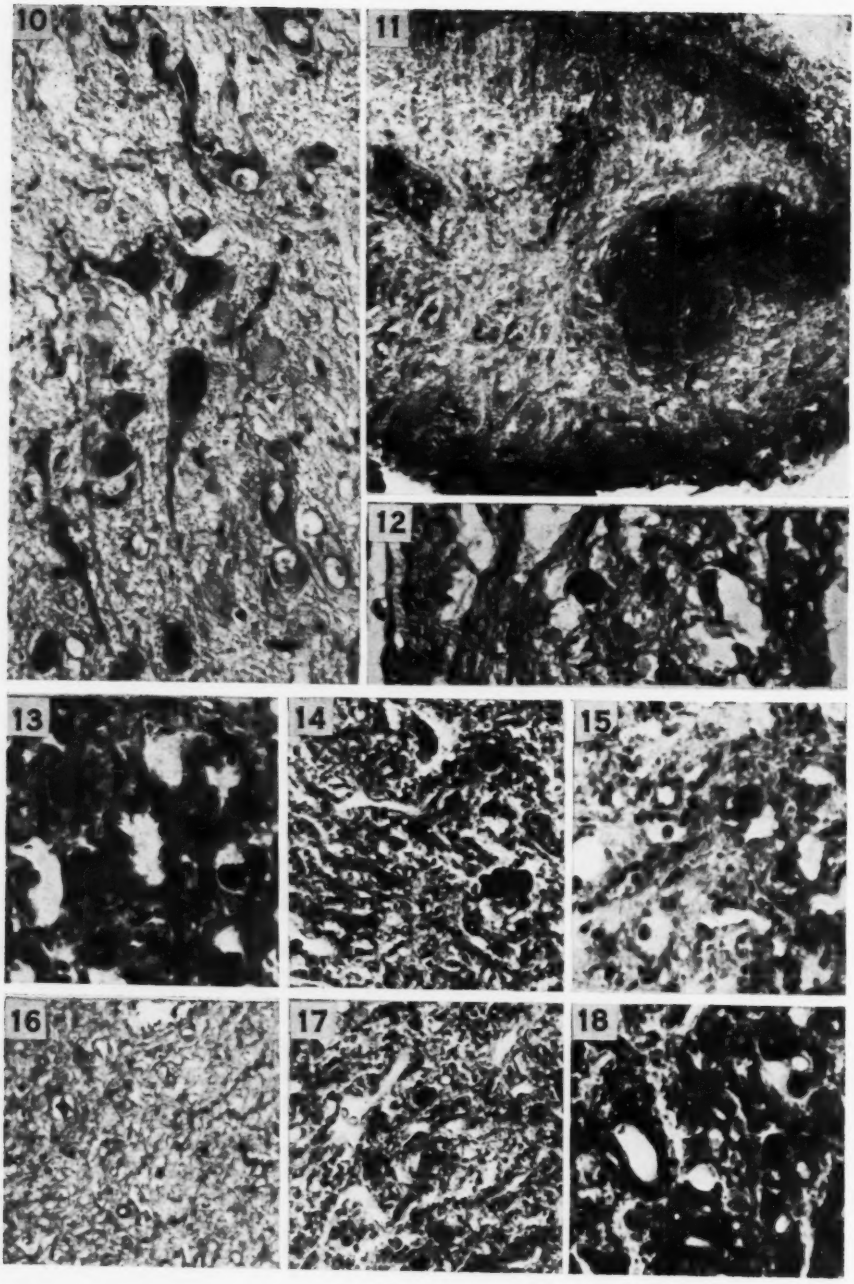

PLATE 2

contained many sulfhydryl and disulfide reactive droplets (Fig. 11). Similar findings occurred in the median eminence of hypophysectomized rats. This was never observed in sections taken from normal animals. Only few observations were made on controls with the selective staining method and these confirm the now well-established results of others concerning the presence of neurosecretory material in the supraoptic neurons and in the hypothalamo-hypophyseal tracts of various animals (Scharrer and Scharrer, 1954).

In vitro and specificity experiments. Five ml. aliquots of either *Pituitrin*, *Pitressin* or *Pitocin*[3] were mixed with 5 ml. of either sublimate-formol, Zenker's fluid or Bouin's fluid. With *Pituitrin*, a heavy precipitate immediately formed; with *Pitressin*, a slight precipitate slowly formed and with *Pitocin* a very slight precipitate gradually formed, except with Bouin's fluid. No precipitate formed with the alcohol and trichloracetic acid mixture. The precipitates which formed with absolute alcohol became soluble when the concentration of alcohol was dropped below 92%. The precipitates formed with the fixatives other than alcohol were insoluble in water, 95% ethyl alcohol, absolute ethyl alcohol, chloroform and xylene. The precipitates formed with Bouin's fluid were somewhat soluble in 80% ethyl

EXPLANATION OF PLATE 2

Figures 13 to 18 are of rat infundibular process of the neurohypophysis. Magnification is ×350 except Figure 16 which is ×600.

FIG. 10. Normal dog hypothalamus. Sublimate-formol. Some of the neurons of the supraoptic nucleus contain material which is reactive for sulfhydryl and disulfide group. Most other hypothalamic neurons contain less.

FIG. 11. Hypothalamus of stalk-sectioned rat. Zenker's fixation. Section through the median-eminence and proximal part of cut stalk (bottom) stained for sulfhydryl and disulfide. Considerable reactive material is present in this section, but was never seen in sections through the same area of normal rats.

FIG. 12. Normal rat infundibular stalk. Zenker's fixation. An isolated globule of sulfhydryl and disulfide reactive material is present.

FIG. 13. Over-hydrated rat (milk diet, 15 days). Sublimate-formol fixation. The sulfhydryl and disulfide reactive material is present in large amounts.

FIG. 14. Overhydrated rat (milk diet, 20 days). Formol-sublimate fixation. Note that the amount of sulfhydryl and disulfide positive material is less than normal.

FIG. 15. Stressed rat. Sublimate-formol fixation. Twenty minutes after a subcutaneous injection of formalin, a depletion of sulfhydryl and disulfide positive material has occurred. Occasional reactive material may be seen in capillaries.

FIG. 16. Stalk-sectioned rat. Zenker's fixation. There is no sulfhydryl and disulfide positive material present. Neurohypophysis appears similar to Figure 1 although the fixative used retains neurosecretory material.

FIG. 17. Stalk-sectioned rat. Zenker's fixation. Some sulfhydryl and disulfide positive material is retained and stains weakly.

FIG. 18. Nornal rat. Zenker's fixation. Figure for comparison with Figures 13–17.

alcohol, especially after long exposure, whereas those formed with other fixatives were insoluble. Attempts at reduction of the disulfides assumed to be present caused the most serious loss of precipitated material. This loss occurred with different reducing agents, and especially with high concentration of them and at the high pH at which the reduction was performed. Thioglycolate, freshly prepared by titrating thioglycolic acid with sodium hydroxide to pH 8 to 8.5, a reducing agent previously used to reduce disulfide groups (Barrnett and Seligman, 1954a), was principally employed in these experiments. When the concentration of thioglycolate which was employed in the histochemical studies (0.25 M for 1 to 2 hours or 0.5 M for 1 hour at 50° C.) was used *in vitro* on the precipitates, *Pitocin* dissolved completely, and only traces of *Pitressin* remained insoluble. Approximately 40 to 50% of *Pituitrin*, precipitated by sublimate-formol, was lost during reduction, over 60% of that precipitated by Zenker's fluid was lost, and of that precipitated by Bouin's fluid, 80 to 90% was lost. There was no further loss of these precipitates during washing with water or 1% acetic acid, or by reacting them with the sulfhydryl reagent, 2,2′-dihydroxy-6,6′-dinaphthyl disulfide (DDD), in an alcoholic buffer solution at pH 8.5. Extraction of the excess of DDD with water, 1% acetic acid, a graded series of ethyl alcohols (70%, 80%, 95% and absolute), acid alcohol and ether also caused no loss of remaining precipitate. Finally, the presence of sulfhydryl groups (free or those derived from reduction of disulfides) was indicated when the reduced and extracted precipitates turned blue after exposure to a freshly prepared solution of tetrazotized diorthoanisidine in phosphate buffer at pH 7.5.

Proof of the specificity of the reactions for the precipitates and for the sections of neurohypophysis was indicated by the absence of color reaction if: (1) the reduced precipitates or the reduced tissue sections were reacted with iodoacetate or N ethyl maleimide before reaction with DDD, or if (2) the precipitates or sections already reacted with DDD were subjected to glutathione before reaction with tetrazotized diorthoanisidine.

The staining reaction was attributed to reduced disulfides and not protein-bound sulfhydryls, because *Pituitrin*, precipitated by absolute acetone and reacted without previous reduction with DDD in acetone and pyridine, failed to stain when tetrazotized diorthoanisidine was added. Under the same condition, the protein-bound sulfhydryl groups of bovine serum albumen, smeared on slides, were stained.

DISCUSSION

The present histochemical observations agree with those of many workers that the neurosecretory material, demonstrable in the neurohypophysis or in the hypothalamo-hypophyseal tracts by selective histo-

logical means, is related to the neurohypophyseal principles obtainable by purification and fractionation of neurohypophyseal extracts (Bargmann, 1949a and b; Bargmann and Hild, 1949; Bargmann, Hild, Ortmann and Schiebler, 1950; Ortmann, 1950, 1951; Bargmann and Scharrer, 1951; Hild, 1951a and b, 1952, 1953; Hild and Zetler, 1951a and b, 1952a and b; Kratzsch, 1951; Scharrer and Scharrer, 1954; Scharrer and Wittenstein, 1952; Smith, 1951; Leveque and Scharrer, 1953; Rothballer, 1953; Palay, 1953; Leveque, 1953).

The basis for this agreement rests upon the presence of histochemically demonstrable disulfide groups in granules and droplets present in the neurohypophysis or in the hypothalamo-hypophyseal system of fibers. This material appears to be the same as the neurosecretory material which is stained with chrome-alum hematoxylin (Bargmann, 1949a), aldehyde-fuchsin (Halmi, 1952) and pinacyanol erythrosinate (Bensely, 1952; Ladman and Barrnett, unpublished observations). The neurosecretory material could not be demonstrated either selectively or histochemically in alcohol-trichloracetic acid fixed tissues in keeping with the observation that neurohypophyseal fractions were soluble in the same fixative. On the other hand, histochemical and selective staining of neurosecretory material occurred after other fixatives, which are better protein precipitates, were used. With these fixatives, a portion of the hypophyseal fractions remained insoluble in *in vitro* experiments and the precipitates were stained in the same manner as the neurosecretory material in tissue sections. However, it should be noted that sometimes the selective staining of neurosecretory material was strongly positive, although the histochemical method was negative. This was probably due to loss of insolubility of the histochemically demonstrable material after Bouin's fluid, loss of material upon washing in 80% alcohol, or loss during reduction. This evidence could also be interpreted as confirming the results of Hild (1951b, 1953) and Schiebler (1951) that the material which was stained selectively was a carrier substance rather than a single substance since, under the conditions enumerated, it could be stained selectively when the disulfides could not be demonstrated. In addition, in the rats which were dehydrated a short time, some of the globules and granules could be recognized even though they did not stain with the histochemical method or stained with phloxine instead of hematoxylin with the selective method.

There was no morphological evidence to indicate that the histochemically demonstrable substance in the neurohypophysis was produced by pituicytes. This is in agreement with the results reported by others for the selective staining method (Ortmann, 1950, 1951; Hild, 1951a and b, 1952; Kratzsch, 1951; Leveque and Scharrer, 1953). In addition, the presence of histochemically stained material in the stalk of the rat and dog, in the hy-

pothalamo-hypophyseal fibers of the dog, and in the median eminence of two stalk-sectioned and the hypophysectomized rats, and the presence of large amounts of disulfide material in the supraoptic neurons of dog and rat, support the numerous observations (Scharrer and Scharrer, 1954) derived from selective staining that the neurosecretory material is produced in hypothalamic neurons and passes along fibers to be released in the infundibular process. The presence of the histochemically stained material in the six vertebrate neurohypophyses examined, suggested that its occurrence is widespread. Indeed, the selectively stained neurosecretory material has been found in every vertebrate to which the method has been applied (Palay, 1953). However, this does not mean that the histochemically and selectively stained materials are necessarily the same, even if they occur within the same morphological structure.

The absence of disulfide-positive material in the neurohypophysis of rats which were dehydrated longest, and its reaccumulation when these animals are allowed water for several days suggest that the material demonstrated histochemically is closely associated with, and may be, antidiuretic hormone. However, the *in vitro* experiments were performed on neurohypophyseal extracts which have antidiuretic activity (Kamm, Aldrich, Grote, Rowe and Bugbee, 1928) and not on pure antidiuretic hormone. In addition, if the disulfide of antidiuretic hormone were demonstrable in the neurohypophyses of normal animals, it is also possible that other proteins which contain a disulfide group may be present. From the *in vitro* experiments, however, there is a direct histochemical parallelism between the material stained in the sections and Pituitrin. The experiments with dehydrated rats, and those which were allowed water after dehydration, confirm the results of others in which the selective staining method was used (Ortmann, 1950; Scharrer, 1952; Hild and Zetler, 1952b; Leveque and Scharrer, 1953).

The findings on examination of the infundibular processes of over-hydrated rats suggest that the maximal amounts of disulfide-positive material which may accumulate during conditions of overhydration that may inhibit the release of antidiuretic hormone are not much greater than the amounts seen in normal animals. In fact, the frequent occurrence of less disulfide-positive material in the infundibular process under this condition may have been due to the stressful nature of the exclusively milk diet on which these rats were kept. The deleterious character of this diet is shown by the fact that some rats not included in these experiments died from it after two weeks. The animals which were purposely stressed by a subcutaneous injection of formalin showed a significant depletion of disulfide material in the neurohypophysis. This result is in agreement with the results described by Rothballer (1953) in which selective staining methods

were used. It is, indeed, attractive to suggest, as Rothballer did, a parallelism between the dissipation of the neurosecretory material in the neurohypophysis after stress, on the one hand, and the simultaneous occurrence of antidiuresis and the release of adrenocorticotropin from the anterior pituitary gland, on the other hand. As antidiuretic hormone is possibly related to neurosecretory material, so a component of the neurosecretory material, though not necessarily antidiuretic hormone, may be the trigger mechanism which excites the release of adrenocorticotropin. The neurosecretory material would then provide a morphological basis for the hypothalamic neurosecretions (Harris, 1948) which are said to effect the functions of the adenohypophysis. However, this speculation is in advance of the evidence at hand. For example, there is an unexplained lack of agreement of experiments in which parts of the hypothalamus have been stimulated or destroyed and which resulted in an effect on adenohypophyseal function. There are nuclear regions in the hypothalamus, the destruction of which exerts an apparent effect on adenohypophyseal function (Barrnett and Mayer, 1954), but which do not contain selectively or histochemically demonstrable neurosecretory material. There is insufficient evidence as to the effects of protein extracts (presumably including neurosecretory material) of the posterior lobe on adenohypophyseal function. On this point, administration of posterior pituitary extracts causes the release of adrenocorticotropin but in doses higher than needed to produce antidiuresis (Nagareda and Gaunt, 1951). There have been no reports describing in detail the isolation and effects of hypothalamic neurohumors. Finally, the manner in which hypothalamic neurohumors or neurosecretory material, if it is a different material, reach the adenohypophysis has not been demonstrated. It has been suggested that both hypothalamic neurohumors (Harris, 1948) and histologically demonstrable neurosecretory material (Palay, 1953) reach the adenohypophysis via the portal blood vessels of the median eminence and infundibular stalk. The general circulation has been invoked by some as a possible route of transport (Hume and Wittenstein, 1950). There is no direct evidence for either of these views. In the observations reported here, there was only a minute amount of histochemically stained material in the median eminence or infundibular stalk in normal rats in comparison to the amount in the infundibular process. The only occasion in which the neurosecretory material was regularly seen within capillaries was in the stressed animals. The histochemically demonstrable material was never seen in the adenohypophysis. In the mouse, but not in the cat, rabbit, guinea pig, rat, monkey and man, the fibers of the hypothalamo-hypophyseal system establish connections with the vessels in the stalk (Palay, 1953) though the two systems approach each other. Nerve endings are regularly encountered in the walls of the blood vessels of the

infundibular process. However, despite the qualification noted above, the role of the neurosecretory material in anterior lobe regulation cannot altogether be dismissed.

The finding that histochemically demonstrable material was present in the neurohypophysis of some of the stalk-sectioned rats is somewhat puzzling. It is possible that the stalk had not been completely transected although the transection was performed under direct vision (Greep and Barrnett, 1951). On the other hand, this result could be interpreted as agreeing with the experiments of Hild and Zetler cited by Scharrer and Scharrer (1954) which showed the slow disappearance of neurosecretory material in transplanted neurohypophyses.

The lack of severe diabetes insipidus in these animals raises an interesting point. In previous work (Greep and Barrnett, 1951; Barrnett and Greep 1951a and b) there seemed to be an inverse relationship between the amount of neurohypophyseal tissue remaining in the proximal stump of the cut stalk and the development of diabetes insipidus. Many authors have noted lack of persistence of diabetes insipidus in stalk-sectioned animals over a period of time. This could be due to reestablishment of connections between the nerves traversing the stalk and the blood vessels of the proximal, cut end of the stalk or the median eminence as indicated by Scharrer and Wittenstein (1952). In the stalk-sectioned animals, as well as in normal animals, it appears that nerve fibers transmitting neurosecretory material establish relationships with blood vessels in the neurohypophysis, which has been said to be part of the hematoencephalic barrier (Wislocki and King, 1936; Wislocki and Leduc, 1952). The sinusoidal capillaries of the neurohypophysis, however, display characteristics different from cerebral capillaries. I would rather suggest that the neurosecretory material travels from very well vascularized areas in the hypothalamus, which are within the blood-brain barrier, to reach equally well vascularized areas of the neurohypophysis which actually may be outside of the blood-brain barrier. In experimental animals which had been given silver nitrate or trypan blue, Wislocki and Leduc (1952) demonstrated that these substances were deposited not only in the perivascular sheaths but also occurred in the tissue spaces and in the parenchymal cells or stromal cells of the neurohypophysis. The great bulk of the brain was unstained by either vital dyes or silver with the exception of certain other small specialized areas which resembled the neurohypophysis. However, they considered the neurohypophysis as part of the blood-brain barrier. Since vital dyes (Cappell, 1929) and silver salts (Hill and Pillsbury, 1939) are said to circulate in the blood in combination with proteins, it would appear that the blood vessels of the neurohypophysis are permeable to this proteinate, whereas brain capillaries are generally impermeable. If the bood-brain barrier is

also a brain-blood barrier capable of operating in the opposite direction, neurosecretions, especially if they are protein in nature, might have to migrate to a region of the brain, such as the neurohypophysis, which is outside the blood-brain barrier, and contains sinusoidal blood vessels rather than cerebral capillaries. In this region, the neurohypophysis, the capillaries presumably would be permeable to neurosecretory material. A similar point of view could be made for the findings in the median eminence of hypophysectomized rats.

The histochemical demonstration of disulfide groups in neurosecretory material and its relationship to neurohypophyseal hormones adds to the list of substances which can be demonstrated by the sulfhydryl and disulfide reaction. These groups occur widely in vertebrate tissues (Barrnett, 1953a; Barrnett and Seligman, 1954a), and have been used to study keratins (Barrnett, 1953a; Barrnett and Seligman, 1954a; Barrnett, Sognaaes and Pettingill, unpublished results). The disulfide reaction has also been used to identify pancreatic enzymes (Barrnett, 1953b), constituents in the acidophils of the adenohypophysis (Ladman and Barrnett, 1954), and insulin (Barrnett and Seligman, 1954b). These experiments indicate the value of using specific histochemical methods in combination with *in vitro* tests, and correlative physiological experiments to indicate the sites of certain protein in tissues.

SUMMARY

The present studies demonstrate that the infundibular processes of the neurohypophyses of normal rats and other animals contain disulfide positive, colloid-like structures which appeared to be the same as neurosecretory material which is stained selectively with chrome-alum hematoxylin. Similar material, positive for disulfide groups, was found in the infundibular stalk of dogs and rats and in the hypothalamo-hypophyseal fibers of the dog. Supraoptic neurons of the rat and dog were richer in disulfide material than other hypothalamic neurons.

In vitro studies on neurohypophyseal extracts revealed that with the histochemical method used, traces of Pitressin and larger amounts of Pituitrin remained insoluble and stained for disulfides. The specificity of the reaction for disulfides of both the staining of tissue sections and of precipitates *in vitro* were performed.

It was not possible to demonstrate the disulfides in acid-alcohol fixed neurohypophyses. Precipitates of neurohypophyseal extracts were also soluble in acid alcohol. It was also shown that the disulfide positive material was depleted from the neurohypophyses of rats after dehydration and reaccumulated on rehydration; that it was depleted upon stress. Upon excessive hydration a variable quantity of disulfide material was present. After

section of the infundibular stalk, some of the rats showed complete absence of the material in the infundibular process and accumulation of large amounts of it in the median eminence. Similar findings occurred in the median eminence of hypophysectomized rats.

The results of these experiments are compared with those of other workers who have used selective staining methods to demonstrate neurosecretory material and the relationship between the histochemically stained material and neurohypophyseal hormones is discussed. Finally, a hypothesis is presented suggesting that the migration of neurosecretory material from hypothalamic neurons to the neurohypophysis may be correlated with differences in the blood vessels within and outside of the blood-brain barrier.

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CORTISONE-INDUCED CONGENITAL CLEFT PALATE IN RABBITS[1]

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WHEN pregnant mice were treated with large doses of cortisone they frequently resorbed their litters (Fainstat, 1951a). Subresorptive doses of cortisone were given and a variety of congenital defects was present among the newborn. Cleft palate was the most frequently visible gross defect. Greatly reduced birth-weights and a very high incidence of stillbirths were evident. The response to cortisone treatment, as measured by the incidence of cleft palate, was markedly influenced by the genetic make-up of the animals, since well-defined differences between mice of different strains were observed in response to identical cortisone treatment (Fraser and Fainstat, 1951; Fainstat, 1953; Fraser, Fainstat and Kalter, 1953).

To learn whether the above phenomena occur only in the mouse, or whether they could apply to other mammals, the following parallel experiments were performed with rabbits.

MATERIALS AND METHODS

Cortisone acetate[3] was injected intramuscularly into the quadriceps in daily doses of 25 and 30 mg. for 4 consecutive days.

The rabbits used in the experiments reported below were albinos bought from a breeder near Montreal who bred "white rabbits" for his livelihood. Their exact genetic constitution is unknown. The animals were fed a standard diet of oats, carrots, lettuce, and water ad libitum, and remained in good health throughout the study.

Each rabbit was kept in a separate cage. The fecundity of the nine bucks which formed our formidable fertilizing force left situations where perhaps more could be desired. Fruition was not always the inevitable aftermath of coitus. At breeding sessions each doe was penetrated and apparently inseminated twice by each of 2 bucks. In spite of the intended thoroughness of the matings, not more than 30% of these does showed evidence of pregnancy when examined near term.

The number of days of pregnancy has in each case been counted from a period 10 hours after mating. It is assumed that rabbit sperm and egg unite at about 10 hours after mating. In no case was a doe permitted to go into labor. Many mammals often eat their dead and abnormal young along with the products of conception. In order to

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[3] Merck "Cortone."

abate distorted interpretation of the experimental findings, total hysterectomy was done some days before the doe was expected to go into labor. Where examination of the uterine contents revealed neither embryos nor placental sites, 2 histological sections of the endometrium were made and studied. The records of such a doe were stricken from more pertinent experimental files if her endometrium did not suggest a recent pregnancy.

Stage of Gestation at Time of Treatment. In similar experiments with mice a 4-day series of cortisone injections begun on the tenth and eleventh day of gestation was most effective in causing cleft palate offspring (Fainstat, 1951a; Fraser and Fainstat, in 1951). The mouse palate usually closes between the fourteenth and fifteenth day of pregnancy. To prevent fusion of the palatine processes, it was anticipated that effective cortisone levels should be present several days before normal palatine fusion occurs. Preliminary embryological studies suggested that the palate is usually closed in the rabbit on the twentieth day of pregnancy, open on day 18, and almost closed on day $18\frac{1}{2}$. Following this apparently successful pattern, cortisone injections in our rabbits were begun on either the fourteenth or fifteenth day of gestation.

RESULTS

Experimental litters

Rabbit T14.—Weight 3.4 kilo. 25 mg. cortisone/day for 4 days, beginning day 15. Hysterectomy day 25. Four living and 7 dead embryos. The dead embryos were about 2 cm. in length and weighed between 1 and 4 gm. each. They appeared much less mature than the live ones, whose crown-rump lengths were about $5\frac{1}{2}$ cm. and which weighed between 10 and 12

TABLE 1. FREQUENCY OF CLEFT PALATE IN OFFSPRING OF DOES GIVEN CORTISONE FOR 4 CONSECUTIVE DAYS DURING PREGNANCY; AND CONTROLS

Does				Embryos				
Identity	Treatment begun	Daily dosage cortisone (mg.)	Litter resorption	Normal palate	Total †C.P.	C.P. alive	C.P. dead	Degen. masses
T14	*D15	25	—	5	6	0	6	0
T23	D14	25	–	3	4	4	0	1
T27	D14	30	–	2	2	1	1	3
T34	D14	30	–	3	5	3	2	0
T16	D15	25	–	5	0	0	0	0
T19	D15	25	+	0	0	0	0	0
T22	D14	25	+	0	0	0	0	+
Totals (Cortisone-Treated Does)			2	18	17			
T25	NIL	0	–	8	0	0	0	0
T30	NIL	0	–	8	0	0	0	0
T31	NIL	0	–	8	0	0	0	0
T32	NIL	0	–	6	0	0	0	0
T39	NIL	0	–	6	0	0	0	0
Totals (Control Does)			0	36	0			

*D = no. days after conception.
† C.P. = median post-alveolar cleft palate.

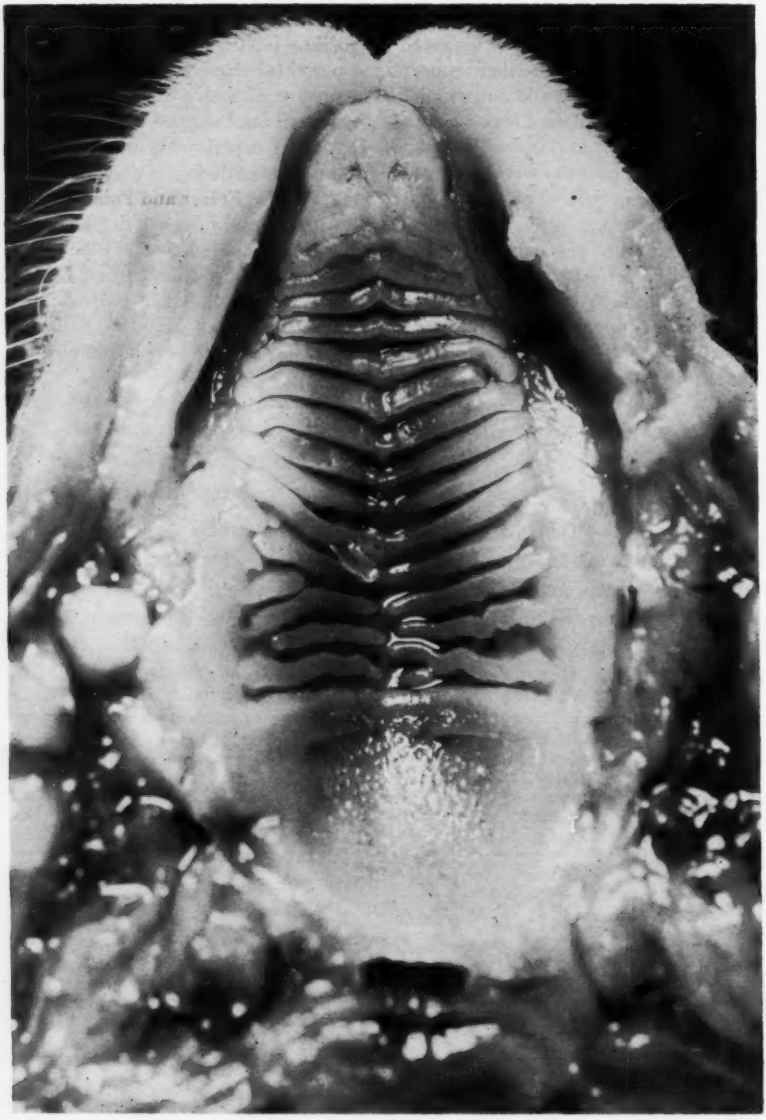

PLATE 1. Palate of a 29-day-old embryo. 30 mg. cortisone per day for 4 days was administered to the mother beginning on the fourteenth day of pregnancy.

PLATE 2. Median post-alveolar cleft palate in a 29-day-old embryo. 30 mg. cortisone per day for 4 days was administered to the mother beginning on the fourteenth day of pregnancy.

gm. each. Six of the 7 dead embryos, and none of the live ones, had cleft palate.

Rabbit T16.—Weight 3.9 kilo. 25 mg. cortisone/day for 4 days, beginning day 15. Hysterectomy day 30. Five grossly normal embryos, normal palates, 37, 37, 35, 35, 32 gm.

Rabbit T19.—Weight 3.4 kilo. 25 mg. cortisone/day for 4 days, beginning day 15. Hysterectomy day 25. No embryos. Histological sections suggest that at least 2 resorbing mesenchyme masses are present in a uterus of decided decidual reaction. This is interpreted as resorption following a true pregnancy.

Rabbit T22.—Weight 5.1 kilo. 25 mg. cortisone/day for 4 days, beginning day 14. Hysterectomy day 28. No embryos. Three dark khaki-brown masses removed from uterine cavity. Histological examination of these masses revealed them to be almost entirely hyalinized, cobwebby in appearance. No tissue type could be identified. The uterus exhibited evidence of decidual reaction. These masses are probably the remains of resorbing embryos and placentae.

Rabbit T23.—Weight 3.0 kilo. 25 mg. cortisone/day for 4 days, beginning day 14. Hysterectomy day 30. One irregularly shaped necrotic mass of tissue (1.5×1 cm.) which was attached to a grossly normal placenta. Seven living embryos: 3 had normal palates, weighed 46, 40, and 37 gm.; 4 had cleft palates, weighed 18, 30, 32, and 34 gm.

Rabbit T27.—Weight 3.8 kilo. 30 mg. cortisone/day for 4 days, beginning day 14. Hysterectomy day 29. Two living embryos, palates "abnormally" arched but not cleft, weighed 22 and 14 gm. One embryo, alive, cleft palate, 12 gm. One embryo dead, necrotic, cleft palate. Three degenerate masses, apparently remnants of placentae and foetuses.

Rabbit T34.—Weight 3.4 kilo. 30 mg. cortisone/day for 4 days, beginning day 14. Hysterectomy day 29. One embryo alive, normal palate 29.5 gm. Two embryos dead, normal palates, weighed 23, 3 gm. Three embryos, alive, cleft palates, weighed 27, 27 and 23.5 gm. Two embryos dead, cleft palates, weighed 4 and 6 gm.

Control litters

Hysterectomies were done on 5 untreated does. Twenty-eight living embryos, all with normal (not cleft) palates, were obtained. In 4 does hysterectomy was done on day 25 and the litters were composed of 8, 8, 8 and 6 embryos respectively. The average weight of these embryos was 18 gm. Six more living embryos with normal palates were obtained after hysterectomy on the twentieth day of pregnancy in the fourth control doe.

DISCUSSION

Other studies

DeCosta and Abelman (1952) studied the effects of cortisone on pregnancy in the rabbit, and no cleft palates were noted following cortisone treatment in 67 pregnant does. However, each of the 67 does received 15 mg. cortisone daily, while we used 25 and 30 mg. Of the 6 does where cortisone treatment was begun on the fifteenth day of pregnancy, 5 aborted after 4, 5 and 6 injections; the sixth doe had 10 young at term. In the remaining experimental does, cortisone injections were begun at a variety of gestational stages, and an unusually high fetal mortality resulted. Courrier *et al.* (1951), and Robson and Sharaf (1952), found that cortisone had a noxious effect on pregnancy, but they did not observe any congenital defects.

Cleft palate and weight of embryos

Seventeen of the 35 embryos recognized in our cortisone-treatment series had a median post-alveolar cleft palate. None of the 36 control embryos had cleft palate. Such a difference is highly significant ($\chi^2 = 22.86$; $P < 0.001$). Also, spontaneous cleft palate in the rabbit has apparently not been reported in the literature.

Embryos with cleft palate almost invariably weighed less than their siblings in the same litter, regardless of whether they were found dead or alive. Consider the litter of T23, where out of 7 live embryos, the 3 with normal palates were each heavier than any of the 4 with cleft palates. Dead embryos weighed less than their living siblings in each litter, as might be expected.

Resorption

Two litters were apparently resorbed, q.v. T19, T22. Among 2 other litters several masses found were believed to be degenerating embryos being resorbed.

Lethal effect

Twelve of the 35 experimental embryos were born dead. Of these 12 embryos, 9 had cleft palate. All of the 36 control newborn were born alive.

A mammalian phenomenon

When cleft palate was found in newborn mice after maternal cortisone treatment, a strong genetic influence was apparent (Fraser and Fainstat, 1951; Fainstat, 1953; Fraser *et al.*, 1953). It will be remembered that one strain of genetically homogeneous mice was relatively susceptible to cortisone treatment, i.e. virtually 100% of the offspring had cleft palate follow-

ing optimum treatment. A different genetically homogeneous strain of mice (Fainstat, 1951b) was relatively refractory to identical treatment. This relationship was maintained throughout the experiments with a variety of doses and stages of gestation. We wondered whether we had struck, by chance, an isolated phenomenon restricted to certain mice. The convincing findings of 17 median post-alveolar cleft palates in rabbits clearly indicates that the phenomenon is not restricted to mice. Furthermore, since the phenomenon occurs in both of the two species adequately investigated to date, it is likely that cleft palate could be produced in many other species if the optimal dosage of cortisone were given at the optimal stage of gestation.

SUMMARY

Median post-alveolar cleft palate may occur in the offspring of rabbits treated with cortisone during pregnancy.

Embryos of subnormal weight and size, and frequently dead, may be found in cortisone-treated pregnant rabbits; in addition, some litters are almost entirely resorbed.

These phenomena have been observed in mice as well, and are thus probably applicable to many other mammals, perhaps inclusive of humans.

Acknowledgments

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A COMPARISON OF THE ANEMIA PRODUCED BY HYPOPHYSECTOMY WITH THAT RESULTING FROM COMBINED THYROIDECTOMY, ADRENALECTOMY AND GONADECTOMY[1]

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EVIDENCE has been presented that the pituitary contains an erythropoietic factor which stimulates red blood cell formation in the rat when given orally or when injected parenterally (Contopoulos, Van Dyke, Simpson, Garcia, Huff, Williams, and Evans, 1953; Van Dyke, Contopoulos, Williams, Simpson, Lawrence, and Evans, 1954; Contopoulos, Van Dyke, Simpson, Ellis, Garcia, Huff, Williams, and Evans, 1953). This pituitary erythropoietic factor is able to repair the post-hypophysectomy anemia, and to prevent the development of the physiological neonatal anemia in the rat (Contopoulos, and Lawrence, 1954). The erythropoietic factor has not been identified with any of the known pituitary hormones and it appears to be a distinct substance (Contopoulos, Van Dyke, Ellis, Simpson, Lawrence, and Evans, 1954; Simpson, Van Dyke, and Williams, 1954).

As the target organs atrophy after hypophysectomy, other workers have directed their attention to the effects of the administration of the target organ hormones themselves (thyroxin, testosterone, and cortisone); they feel indeed, that these hormones repair the post-hypophysectomy anemia and that it is unnecessary to postulate a separate pituitary erythropoietic factor (Crafts, 1949).

It is the object of this paper to compare the anemias, in both rate of development and severity, which ensue after hypophysectomy and after ablation of those endocrine organs which show marked atrophy after hypophysectomy, namely of the thyroids, adrenals and gonads.

MATERIAL AND METHOD

Male rats of the Long-Evans strain were hypophysectomized, using the parapharyngeal approach on the 26–28th day of life. The completeness of hypophysectomy was de-

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termined at autopsy by examination of the sella turica and histologically by the atrophy of the pituitary target organs. No data from animals found to be incompletely hypophysectomized were included in the results.

The thyroid was removed surgically and the completeness of thyroidectomy was determined at the end of the experiment by the uptake of I^{131}. A known dose of I^{131} was injected intravenously 24 hours before autopsy. At autopsy the trachea and the entire ventral neck region were removed and the radioactivity was counted in a scintillation counter (Anger, 1951). This radioactivity was compared with that of a mass of muscle approximately the same size taken from the leg of the same animal. The difference in the counts was ascribed to any thyroid remnants present. By comparing the count from the ventral neck region of experimental animals with that from normal rats which had received the same dose of I^{131} the per cent of thyroid remaining was calculated. All the animals which had an I^{131} uptake in the neck region greater than 2.0% of normal were considered incompletely thyroidectomized and data from them were not included in the results.

Adrenalectomy was performed through the lumbar approach and all adrenalectomized rats were given 1% NaCl in the drinking water. At autopsy the site of the adrenals was inspected in order to exclude the presence of adrenal remnants. Completeness of adrenalectomy has been shown to be 90–95% (Van Dyke, Contopoulos, Williams, Simpson, Lawrence, and Evans, 1954).

Orchidectomy was done in the usual way through a low median abdominal incision. The three types of organs were removed under the same ether anesthesia. All animals received, on two consecutive days following operation, 0.5 cc. of 0.5% terramycin dissolved in a 5% glucose solution.

Body weight changes were followed in all animals, and depression of growth rate was of assistance in determining completeness of the thyroidectomy and hypophysectomy. The basal metabolic rate was determined at intervals in all groups using the method described by Kleiber (1932, 1951).

All animals were maintained on a standard laboratory diet (Fortgang and Simpson, 1953) given *ad libitum*, with a supplement of fresh lettuce once a week and were kept in a constant temperature room throughout the experiment ($75 \pm 2°$ F.).

The total circulating red cell volumes were determined by the Fe^{59} labeled cell dilution method (Berlin, Van Dyke, Siri, Williams, 1950). The method was briefly as follows: The labeled cells were obtained from a Long-Evans donor rat previously injected with Fe^{59}. The experimental animals were injected through the saphenous vein with 0.1 ml. of donor blood containing approximately 0.03 μc Fe^{59}. After allowing 6 minutes for the blood to mix, a sample of blood was drawn from the external jugular vein into a heparinized syringe. A known volume of this blood was pipetted into a vial and its activity counted directly in a scintillation counter (Anger, 1951). The total blood volume was calculated from the fraction of the injected activity found in the sample of known volume. The hematocrits were determined in Wintrobe tubes. The total blood volume thus calculated, multiplied by the hematocrit, gave the total circulating red cell volume. The values for the total circulating red cell volumes were then divided by the values for the body weights of the animals and the results are presented in terms of ml. of red blood cells per 100 gm. body weight.

The hemoglobin concentration was determined by the method of Turner (1946) and the results are presented as gm./100 ml. The total blood volume multiplied by the hemoglobin concentration gave the total circulating hemoglobin. The total circulating hemoglobin was then adjusted for the body weight of the animal and the results are presented in terms of gm./100-gm. body weight.

RESULTS AND DISCUSSION

As stated, the completeness of hypophysectomy or combined thyroidectomy, adrenalectomy and orchidectomy was judged by the depression of rate of body weight increase, by the depression of the basal metabolic rate, and the I^{131} uptake of the thyroid region, and finally, by the absence of fragments of the endocrine glands at sites from which they were removed. Only data from animals satisfying the above criteria were included in the results. Figure 1 represents the body weight increases of the normal rats and those subjected to hypophysectomy or ablation of the three organs.

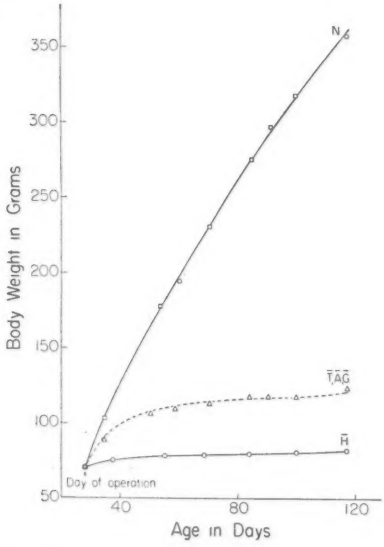

FIG. 1. Body weights of normal, hypophysectomized and thyroidectomized-adrenalectomized-gonadectomized male rats.

It can be seen that the animals from which the thyroid, adrenals and testes were removed showed some increase in body weight for approximately 20 days postoperatively; after this, growth was negligible. Practically no growth occurred in the hypophysectomized rats after operation.

Table 1 represents the average basal metabolic rate, and the I^{131} uptake of the ventral neck tissues removed from the different groups. It can be seen from the table that the average basal metabolic rate of animals subjected to the triple operation was at least 50% below normal. Approximately the same per cent decrease followed hypophysectomy. The I^{131} uptake of rats subjected to the combined operation was only 0.4% of the normal uptake. This is considered excellent evidence of the adequacy of the

TABLE 1. BASAL METABOLIC RATE AND I^{131} UPTAKE OF NORMAL RATS AND THOSE SUBJECTED TO HYPOPHYSECTOMY OR COMBINED THYROIDECTOMY, ADRENALECTOMY AND GONADECTOMY

Groups	BMR	I^{131} uptake
	Cal./m.²/hr.	per cent
Normal	45.0 ± 2.0*	100.00
Hypophysectomized	23.3 ± 1.4.	18.0 ± 1.0
Thyroidectomized, Adrenalectomized, Gonadectomized	22.7 ± 1.5	0.4 ± 0.1

* Standard Error = $\sqrt{\frac{\Sigma d^2}{n(n-1)}}$.

thyroidectomy, especially when one takes into account the 18% uptake of I^{131} which characterized hypophysectomized rats.

Figure 2 shows graphically a comparison between the anemias developing after hypophysectomy and after combined thyroidectomy, adrenalectomy and gonadectomy. Three sets of curves show respectively the decreases in hematocrit, hemoglobin and red cell volume in the two groups (Fig. 2.) Table 2 presents in detail the hematological values for the normal and for the experimental groups at progressively longer intervals after the

TABLE 2. HEMATOLOGICAL VALUES OF THYROIDECTOMIZED, ADRENALECTOMIZED, AND GONADECTOMIZED RATS AS COMPARED WITH THOSE OF NORMAL AND HYPOPHYSECTOMIZED RATS

Days after operation	Group	No. of rats	Weight	Hgb.	Hgb/100 gm. body weight	Hematocrit	Red cell volume per 100 gm. body weight
			gm.	gm./100 ml.	gm.	per cent	ml.
0		6	69 ± 2*	13.5 ± 0.1	0.70 ± 0.02	46.1 ± 1.2	2.38 ± 0.03
17	N	6	160 ± 7	12.9 ± 0.1	0.70 ± 0.02	46.0 ± 0.9	2.42 ± 0.02
	$\overline{T}+\overline{A}+\overline{G}$	5	105 ± 6	12.9 ± 0.2	0.64 ± 0.02	45.0 ± 0.08	2.22 ± 0.04
	$\overline{H}$	6	73 ± 2	12.3 ± 0.1	0.62 ± 0.02	44.5 ± 0.08	2.22 ± 0.04
29	N	5	213 ± 6	12.7 ± 0.1	0.65 ± 0.03	46.8 ± 0.7	2.36 ± 0.04
	$\overline{T}+\overline{A}+\overline{G}$	5	107 ± 4	11.5 ± 0.3	0.54 ± 0.01	44.9 ± 0.5	2.08 ± 0.03
	$\overline{H}$	6	75 ± 3	10.6 ± 0.1	0.52 ± 0.01	41.5 ± 0.5	2.03 ± 0.02
40	N	4	254 ± 8	12.6 ± 0.2	0.65 ± 0.03	46.0 ± 0.6	2.37 ± 0.03
	$\overline{T}+\overline{A}+\overline{G}$	5	108 ± 6	11.5 ± 0.2	0.55 ± 0.04	42.1 ± 0.8	2.00 ± 0.05
	$\overline{H}$	6	81 ± 3	9.8 ± 0.3	0.48 ± 0.03	38.0 ± 0.4	1.84 ± 0.03
77	N	5	358 ± 10	14.7 ± 0.2	0.73 ± 0.03	47.1 ± 0.9	2.36 ± 0.04
	$\overline{T}+\overline{A}+\overline{G}$	5	136 ± 3	13.2 ± 0.0	0.65 ± 0.03	39.4 ± 1.0	1.92 ± 0.06
	$\overline{H}$	7	92 ± 3	9.8 ± 0.3	0.43 ± 0.03	29.8 ± 0.4	1.30 ± 0.04

N = Normal male rats.
$\overline{T}+\overline{A}+\overline{G}$ = Thyroidectomized, adrenalectomized, gonadectomized male rats.
$\overline{H}$ = Hypophysectomized male rats.

* Standard Error = $\sqrt{\frac{\Sigma d^2}{n(n-1)}}$.

operations. It can be seen that the hypophysectomized rats showed a severe and progressive anemia as compared with the normal rats. The animals subjected to the triple operations developed a milder anemia, which was not progressive in severity. Seventy-seven days after operation a 46% drop in red cell volume had occurred in the former group, whereas approximately 19% decrease had occurred in the latter group. A decrease in the hematocrit and hemoglobin was observed in both groups but was more severe and progressive in the hypophysectomized rats.

Crafts (1953) reported recently that, following thyroidectomy and adrenalectomy, adult Wistar female rats showed a 17% decrease in erythrocyte count, 26% decrease in hemoglobin, and 26% decrease in hematocrit. The hemoglobin and hematocrit values reported here from study of rats of the Long-Evans strain have been reduced less after combined thyroidectomy, adrenalectomy and gonadectomy, the decreases being 10% and 16% respectively. In hypophysectomized rats Crafts reported a de-

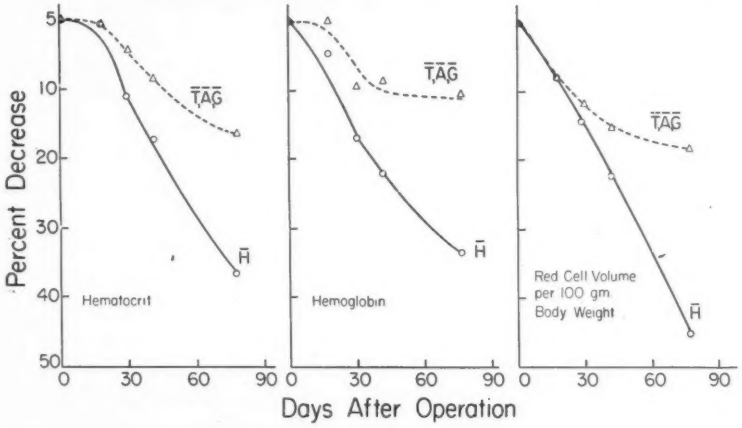

FIG. 2. Per cent decrease in hematocrit, hemoglobin and red cell volume per 100 gm. body weight following hypophysectomy as compared with simultaneous thyroidectomy, adrenalectomy and gonadectomy in male rats.

crease of only 19% in red cell count, a decrease of 29% in the hemoglobin concentration and a decrease of approximately 30% in the hematocrit. This is in contrast with the severity of anemia which develops in the Long-Evans strain of rat, as judged by red cell volume determined by the Fe^{59} labeled cell method. The red cell volume, as reported in this study, decreased at least 45% by the 77th day, the hematocrit 37%, and the hemoglobin 35%. It is possible that the discrepancy in the anemias may be due to the difference in the post-hypophysectomy interim required by the two

strains of rats to develop anemia. However, it must also be borne in mind that results based largely on erythrocyte counts may be subject to error introduced from hemoconcentration which occurs after hypophysectomy (Berlin, Van Dyke, Siri, Williams, 1950; Contopoulos, Lawrence, Simpson, In Preparation), whereas results based on red cell volume are not subject to this distortion.

If it were true that the post-hypophysectomy anemia is due to the atrophy of the target organs, then the triply-operated animals should have as severe an anemia as the hypophysectomized animals. However, no such severe anemia had developed in the triply-operated animals 77 days after operation. Thus, the lack of the target organ hormones due to the atrophy of these organs after hypophysectomy does not completely explain the post-hypophysectomy anemia. This is in accordance with the observation already reported, that the anemia resulting from simultaneous absence of the thyroids, adrenals and gonads is made more severe by removal of the hypophysis (Van Dyke, Contopoulos, Williams, Simpson, Lawrence, and Evans, 1954).

The mild anemia which is present in thyroidectomized, adrenalectomized and orchidectomized rats may be explained in part at least by secretory changes in the pituitary resulting from absence of these target organs. The lack of the hormonal secretion of the target organs results in histological and physiological changes in the pituitary. Among the physiological changes may be a reduced production or secretion of the pituitary erythropoietic factor. Evidence has been presented elsewhere that there is a pituitary erythropoietic factor specifically stimulating erythropoiesis and that injections of pituitary extracts can repair the post-hypophysectomy anemia (Contopoulos, Van Dyke, Simpson, Garcia, Huff, Williams, and Evans, 1953; Van Dyke, Contopoulos, Williams, Simpson, Lawrence, and Evans, 1954; Contopoulos, Van Dyke, Simpson, Ellis, Garcia, Huff, Williams, and Evans, 1953; Contopoulos, Van Dyke, Ellis, Simpson, Lawrence, and Evans, 1954; Simpson, Van Dyke, and Williams, 1954).

SUMMARY

Thyroidectomized, adrenalectomized, orchidectomized male rats developed a moderate anemia as compared to the severe and progressive anemia of the hypophysectomized rat. These observations are interpreted as strengthening the hypothesis that the pituitary produces an erythropoietic factor necessary for normal erythropoiesis.

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NOTES AND COMMENTS

THE EFFECT OF SODIUM-RETAINING CORTICOID, ELECTROCORTIN, DESOXYCORTICOSTERONE, AND CORTISONE ON RENAL FUNCTION AND EXCRETION OF SODIUM AND WATER IN ADRENALECTOMIZED RATS

A sodium-retaining corticoid, extracted from the urine of edematous patients with nephrosis and heart failure (Deming and Luetscher, 1950), has been isolated by chromatography (Luetscher and Johnson, 1953, 1954) and has been observed to behave in many respects like preparations of the sodium-retaining hormone from adrenal cortical extract (Simpson and Tait, 1953; Mattox, Mason and Albert, 1953; Knauff, Nielson and Haines, 1953). These preparations from adrenal cortical extract and from urine have been assayed in adrenalectomized rats (methods reviewed by Dorfman, 1953) and showed a potency 20 to 100 times as great as desoxycorticosterone acetate (DCA).

Since the mechanism of sodium conservation by these new hormones has been in doubt, measurements have been made on the effects of adrenalectomy, cortisone, DCA sodium-retaining corticoid, and electrocortin on renal function in rats under the conditions used in bioassay.

METHODS

In each experiment, 10 or 12 female rats weighing 150 gm. were studied simultaneously, one half of the group receiving the material to be tested, the other half receiving only the solvent (95% ethanol, .05 ml.). The adrenalectomized rats were prepared as in the bioassay for sodium-retaining activity (Leutscher and Deming, 1951; Johnson, 1954). Urine was collected for 5 hours after the simultaneous administration of 5 cc. of water intraperitoneally and the test substance intramuscularly. The rats were sacrificed at the end of one day on experiment, when blood was collected from each rat. Creatinine in serum and urine was measured by the method of Bonsnes and Taussky (1945). The rate of excretion (urine volume, UV) and clearance (CV/P(lasma) per 100 gm. body weight) of endogenous creatinine were calculated. Sodium and potassium were measured by flame photometry. Sodium output is expressed as the square root (Table 1) for statistical reasons (Johnson, 1954).

Crystalline electrocortin was prepared by Simpson and Tait (1953). The sodium-retaining fraction was prepared from the urine of a child with nephrotic syndrome by the method of Luetscher and Johnson (1954). Crystalline DCA and cortisone were gifts from Ciba Pharmaceutical Products and from Merck and Co.

RESULTS

The results and analysis of variance are summarized in Table 1. Under the experimental conditions, adrenalectomy was followed by a reduction of creatinine clearance impaired diuresis, and increased loss of sodium in the urine with reduced serum sodium concentration. The rats with intact adrenals (sham operated) showed a range of 141 to 145 mEq. sodium and 4.4 to 5.6 mEq. postassium/L. of serum. The adrenalectomized animals had levels of 129 to 133 mEq. sodium/L. and 6.9 to 7.3 mEq. potassium/L., which were not affected significantly by the one small dose of hormone given.

Cortisone in a dose of 100 μg. increased diuresis and creatinine excretion. The high sodium output was not decreased by cortisone.

Ten microgram doses of DCA increased the output of creatinine, while sodium excretion declined significantly

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TABLE 1. EFFECTS OF ADRENALECTOMY AND TREATMENTS ON RENAL FUNCTION

	No. expts.	Total rats	Urine volume, μl./min.	Total Na, μEq.[1/2]	Total K, μEq.	Creatinine	
						UV,[1] μg./min.	UV/P, μl./min.
Adrenalectomized	1	4	6.1	14.7	68	2.40	319
			5.8, 11.6	*2.4, 10.1*			*6, 236*
Sham operated		5	15.8	8.4	65	3.02	440
Adrenalectomized	2	9	8.7	10.2	136	2.76[i]	341[i]
			0.5, 6.5			*.23, .83*	
Adrenalectomized +100 μg. cortisone		10	12.3	12.5	155	3.28	353
Adrenalectomized	4	18	8.0[e]	11.3[e]	94[e]	2.74[e]	327
				2.6, 5.7		*.02, .61*	
Adrenalectomized +10 μg. DCA		24	6.7	7.8	107	2.94	356
Adrenalectomized	2	9	7.9[e]	8.2[e]	134	2.60	319
				1.0, 3.7			
Adrenalectomized +0.4 μg. SRC		10	8.0	5.8	134	2.84	316
Adrenalectomized	1	4	7.8	9.4	133	2.78	338
				2.5, 5.0			
Adrenalectomized +0.2 μg. electrocortin		6	8.2	5.6	120	2.67	298

Underline = Response significant.
Italics = Confidence limits (95%) of difference of means.
[e] = Experiments differ significantly.
[i] = Interaction significant.
[1] Urine volume.

The dosage of sodium-retaining corticoid in the crude preparation used was approximately 0.4 μg. A reduction in sodium output was observed without significant alteration of creatinine output or clearance.

The effects of 0.2 μg. electrocortin resembled those of 0.4 μg. sodium-retaining corticoid; sodium output was reduced without significant effect on creatinine excretion. Neither substance affected urine volume. A marked increase in urine volume was obtained with 100 μg. cortisone whereas a modest decrease was usually noted after 10 μg. DCA discussion.

DISCUSSION

These results agree with the observations of Desaulles, Tripod, and Schuler (1953) in showing what electrocortin has a greater effect on sodium retention than on potassium or water excretion in adrenalectomized rats.

The clearance of creatinine in the rat is considered to measure glomerular filtration rate (GFR), (Friedman, 1947). The normal rat can increase GFR as much as three-fold after large loads of fluid, while the adrenalectomized rat responds to similar loading with a smaller increase of GFR and a far smaller output of urine (Friedman, 1947; Boss, Birnie, and Gaunt, 1949). The moderate water load used in the present experiments resulted in intermediate levels of GFR and of urine flow in sham-operated rats, in con-

formity with Friedman's observations on normal rats. GFR and diuresis in the adrenalectomized rats were significantly subnormal.

SUMMARY

Under the conditions used in the bioassay of sodium-retaining activity, adrenalectomized rats showed subnormal glomerular filtration rate, impaired diuresis, and loss of sodium in the urine with reduced serum sodium concentration.

Cortisone (100 μg.) increased creatinine output and diuresis, but failed to reduce sodium loss.

DCA (10 μg.) increased creatinine output and reduced sodium loss; but urine flow was decreased. Sodium-retaining hormone from urine and from adrenal cortex (electrocortin), in doses of a fraction of a microgram, reduced sodium loss without affecting glomerular filtration rate or diuresis. These sodium-retaining hormones appear to affect primarily the reabsorption of sodium by the renal tubules.

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AWARDS OF THE ENDOCRINE SOCIETY

The selection of the recipients of the awards of The Endocrine Society is made by a Committee appointed by the Council of the Society. These awards and fellowships carry no obligation by the recipient to the Society or to the donors.

Medal of The Endocrine Society

In 1954 the Council of the Society voted to establish a medal and an honorarium of $1,000 to be given to an individual for work of special distinction in endocrinology. The recipient shall be chosen from nominations presented by members of the Society and is limited to citizens of the United States and Canada.

The Ciba Award

The Ciba Award to recognize the meritorious accomplishments of an investigator not more than 35 years of age in the field of clinical or preclinical endocrinology was established in 1942, but no recipient was selected in 1942 or 1943. In 1944 the Award was presented to Dr. E. B. Astwood; 1945, Dr. Jane A. Russell; 1946, Dr. Martin M. Hoffman; 1947, Dr. Choh Hao Li; 1948, Dr. Carl Heller; 1949, Dr. George Sayers; 1950, Dr. Oscar M. Hechter; 1951, Dr. Albert Segaloff; 1952, Dr. Seymour Lieberman; 1953, Dr. Sidney Roberts and Dr. Claire Szego (Mrs. Roberts); 1954, Dr. Isodore M. Rosenberg. Prior to 1952 the Award was $1,200. It is now $1,800. If within twenty-four months of the date of the Award, the recipient should choose to use it toward further study in a laboratory other than that in which he is at present working, it will be increased to $2,500.

The Ayerst, McKenna and Harrison Fellowship

This fellowship was established in 1947. It is designed to assist men or women of exceptional promise in furthering their advancement towards a career in endocrinology. The fellowship is awarded on alternate years (1955, 1957, etc.) and the stipend which will not exceed $5,000 may be divided into two Fellowships in varying amounts in accordance with the qualifications of the appointees. Individuals possessing the M.D. or Ph.D. degree or candidates for either of these degrees are eligible for appointment.

Applicants must submit the following information:

(1) Evidence of scientific ability as attested by studies completed or in progress.

(2) Recommendations from individuals familiar with the candidate and his work.
(3) A proposed program of study.
(4) Acceptance of the individual by the head of the department in which the Fellowship will be held.
(5) A statement that he or she will serve full time if awarded a Fellowship. A small amount of time (10 to 15 per cent) may be spent in course work or participation in teaching, the latter purely on a voluntary basis.

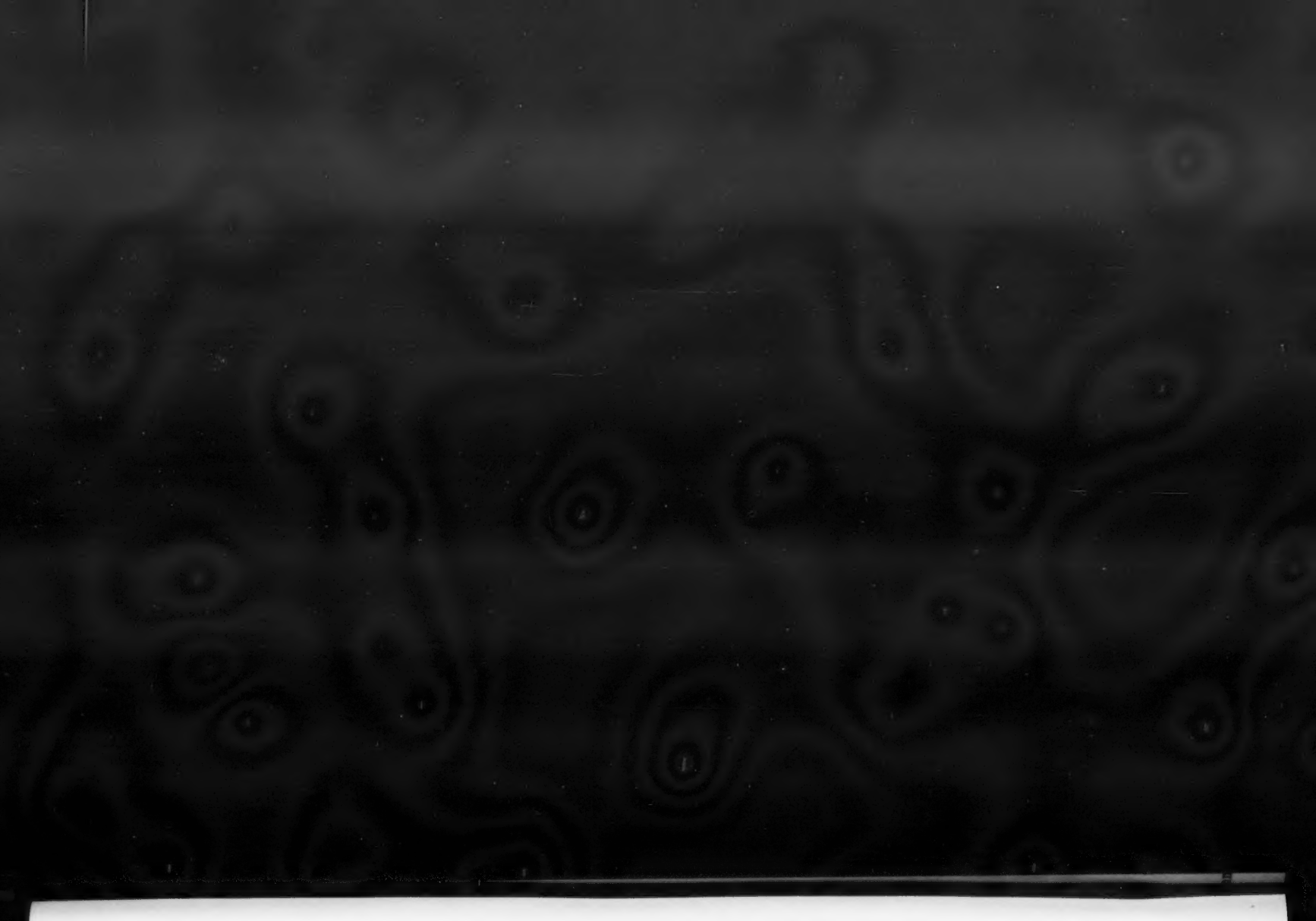
The Schering and The Upjohn Scholars of The Endocrine Society

The Council of The Endocrine Society established a category of Scholars. These grants have been made available through the generosity of The Schering Corporation and The Upjohn Company, and will be awarded to established investigators and teachers in the fields of endocrinology who wish to extend their opportunities for work either in this country or abroad.

The award will not exceed $2,500 annually for each individual, and shall be granted on the basis of proposals submitted by the applicant and such applications should include the estimated financial needs. The funds may be used for travel, maintenance and other expenses.

Nominations

Nominations for the Medal of The Endocrine Society; the Ciba Award; and the Ayerst, McKenna and Harrison Fellowship may be made by any member of The Endocrine Society. They should be submitted on forms which may be obtained from the Office of the Secretary, 1200 N. Walker Street, Oklahoma City 3, Oklahoma. Completed nominations should be returned to the Secretary not later than November 1st each year.

Proposals for appointment as Scholars of The Endocrine Society should be made in writing by the individual, and addressed to the Secretary of the Society. They should be submitted by November 1, 1954.

The Awards Committee will meet in November and notice of awards to successful nominees and applicants will be made not later than December 1, 1954.